

NBS

Technical Note

274

ORGANIC CHEMISTRY: RADIOACTIVE CARBOHYDRATES, SUGARS
IN SOLUTION, ALDOL CONDENSATIONS, MOLECULAR STRUCTURE,
SYNTHESIS OF SELECTED COMPOUNDS, AIR POLLUTION STUDIES,
REFERENCE MATERIALS (ORGANIC)

JULY 1964 TO JUNE 1965



U. S. DEPARTMENT OF COMMERCE
NATIONAL BUREAU OF STANDARDS

THE NATIONAL BUREAU OF STANDARDS

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NATIONAL BUREAU OF STANDARDS

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ISSUED DECEMBER 3, 1965

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Edited by Horace S. Isbell
Organic Chemistry Section
Analytical Chemistry Division
Institute for Materials Research

NBS Technical Notes are designed to supplement the Bureau's regular publications program. They provide a means for making available scientific data that are of transient or limited interest. Technical Notes may be listed or referred to in the open literature.

FOREWORD

The Analytical Chemistry Division was established as a separate division at the National Bureau of Standards on September 1, 1963, and became part of the Institute for Materials Research in the February 1, 1964, reorganization. It consists at present of seven sections and about 85 technical personnel encompassing some 30 different analytical competences from activation analysis and atomic absorption to vacuum fusion and x-ray spectroscopy. These competences, and in turn the sections which they comprise, are charged with research at the forefront of analysis as well as awareness of the practical sample, be it standard reference material or service analysis. In addition it is their responsibility to inform others of their efforts.

Formal publication in scientific periodicals is highly important. In addition, however, it has been my experience that informal, annual summaries of progress describing efforts of the past year can be very valuable in disseminating information. At the National Bureau of Standards such publications fit logically into the category of Technical Note. In 1965 we plan to issue these summaries for all of our sections. The following is the first annual report on progress of the Organic Chemistry Section.

W. Wayne Meinke, Chief
Analytical Chemistry Division

PREFACE

The Organic Chemistry Section originated in a combination, in 1948, of the Polarimetry Section of the Optics Division and the Organic Chemistry Section of the Chemistry Division. In the reorganization of 1960, the Section was assigned to the Physical Chemistry Division, which became part of the Institute of Basic Standards in the reorganization of February 1, 1964. The Section was transferred to the Analytical Division of the Institute for Materials Research on February 17, 1965. This report covers the principal activities of the Section in both Institutes from January 1, 1964 to July 1, 1965. Considerable background material is presented for exposition of the overall program.

Most of our activities arise from need, on the part of the scientific public, for assistance on problems within the scope of the Bureau's mission, within the area assigned to the Division and Section and within the special competence of the staff. Certain activities have goals, largely determined by the needs of the public. Thus, the primary goal of the ^{14}C -labeled carbohydrate project is to make urgently needed, labeled carbohydrates available to the scientific public; the goal of the metallo-organic standards project is to provide suitable standards for determination of metals in lubricating oils; the goal of our phase of the air pollution project is to obtain knowledge on the oxidation of

hydrocarbons that occur in polluted air. We conduct a vigorous research program in order to maintain competence in the ever-increasing number of specialized techniques of organic chemistry. By so doing, we are able to support the over-all Bureau program and render aid to the scientific public. In selecting projects, preference is given to problems of wide applicability and to the development of basic principles. We prefer projects which yield methods of measurement, or techniques applicable to a variety of problems. We carefully avoid merely extending methods or techniques that follow known patterns. In the sections that follow, some of the current and recently completed activities of the staff are described briefly.

In order to describe experimental procedures adequately, it has been necessary occasionally to identify commercial materials and equipment in this report. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

Horace S. Isbell, Chief
Organic Chemistry Section

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July 1964 to June 1965

Edited by Horace S. Isbell

ABSTRACT

The report describes work in progress in the Organic Chemistry Section of the Analytical Chemistry Division at the National Bureau of Standards. It presents reviews of recent contributions of the Section on the following subjects: Synthesis of carbon-14- and tritium-labeled carbohydrates, isotope dilution and double-label methods of analysis, determination of kinetic isotope effects, use of solvent isotope-effects for studying pyranose-furanose interconversions, measurements of rates of primary enolization, rates of rearrangement of one sugar to another by bases, estimation of the relative stabilities of isomeric hexoses, reversible aldol condensations, mechanism for the formation of saccharinic acids, formation of branched-chain aldoses and linear ketoses by aldol condensations, stereochemistry of monoaminotetrahydroxycyclopentane derivatives, cyclic polyhydroxy ketones, phenylhydrazono-phenylazo tautomerism, acetamido-deoxyketoses, syntheses and properties of selected organic compounds, interaction of aromatic hydrocarbons with oxygen, oxidation of polycyclic, aromatic hydrocarbons on particulate matter, loss on filtration of aqueous solutions of polycyclic, aromatic hydrocarbons, oxidation products of pyrene, and preparation of 1-phenyl-1,3-butanedione chelates of chromium and iron for use as new metallo-organic standards.

Key Words

Radioactive carbohydrates
Carbon-14-labeled carbohydrates, synthesis of
Glucose-3-C-14
Tritium-labeled carbohydrates, synthesis of
Isotope dilution techniques
Double-label method of analysis
Cyanohydrin synthesis
Reduction of aldonic lactones
Liquid scintillation counting
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Photochemical changes in thin-layer chromatograms
Adsorption of hydrocarbons on filters
Oxidation products of pyrene
Separation of pyrenediones by column chromatography
Reference materials, organic
Metallo-organic compounds

1. RADIOACTIVE CARBOHYDRATES

(H. L. Frush, N. B. Holt, H. S. Isbell,
L. T. Sniegowski, and C. W. R. Wade)

A. Synthesis of Carbon-14-labeled Carbohydrates

1. General Methods

For a number of years, the Organic Chemistry Section has carried out an extensive program on the development of methods for synthesizing and utilizing carbohydrates position-labeled with carbon-14 or tritium. These urgently needed radioactive compounds have been supplied to nearly every major biological research laboratory in the country and to several laboratories abroad. Methods of synthesis and analysis have been described in approximately 40 published papers, and over 2500 samples have been supplied to other research workers. (Some of the methods are given in references 1 to 8, inclusive.) Gradually, as commercial laboratories have become able to synthesize radioactive carbohydrates (frequently, by our published methods), the Section has discontinued supplying them, except under special circumstances (as, for instance, when commercial supplies have been temporarily depleted). The Section does conduct certain difficult syntheses of radioactive carbohydrates when (a) there is a demonstrable need for a compound; (b) the compound, if available, would have numerous applications in research; and (c) commercial production of the compound is not feasible.

In order to prepare a radioactive sugar position-labeled

with carbon-14, it is necessary to begin with a sugar having one less carbon atom in the chain, and to extend the chain by a series of reactions, starting with the addition of a ^{14}C -labeled reagent. The reaction that we chose for adaptation to the semimicro scale necessary in the synthesis of a $1\text{-}^{14}\text{C}$ -labeled sugar is the so-called Kiliani synthesis [9]. It begins with the addition of cyanide (cyanohydrin reaction) as indicated below:

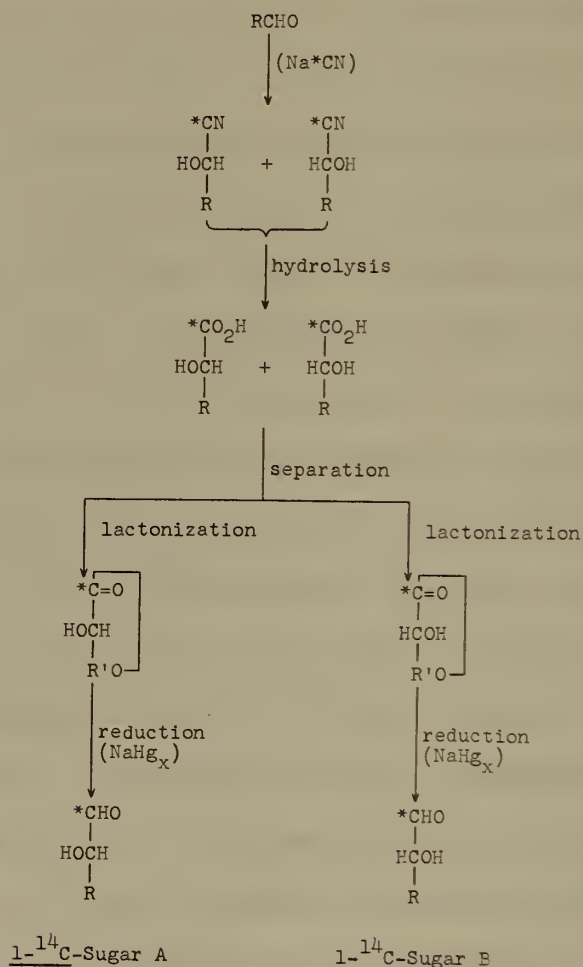


Figure 1. Synthesis of a $1\text{-}^{14}\text{C}$ -labeled sugar.

Addition to the carbon chain creates a new asymmetric center and affords two compounds. At some stage in the subsequent processes, these two epimers (diastereoisomers differing in configuration at C-2) must be separated. This has usually been accomplished on the semimicro scale by co-crystallization with the non-labeled (carrier) compound. Addition of the carrier reduces the specific activity of the compound separated. For the synthesis of 1-¹⁴C-labeled sugars, the diastereoisomers are usually separated as the salts of the corresponding aldonic acids.

For the preparation of 2-¹⁴C-labeled sugars, a 1-¹⁴C-sugar is allowed to react with non-labeled cyanide in a second Kiliani synthesis. This synthesis again creates epimers, thus drastically lowering the yield of the desired compound. Furthermore, the separation of epimers by co-crystallization with the non-labeled form again lessens the specific activity. Nevertheless, the Section has developed methods for the preparation of several 2-¹⁴C-labeled sugars [10]; some of these are now commercially available.

2. D-Glucose-3-¹⁴C

A procedure for the synthesis of this compound has recently been developed [11]. In order to synthesize D-glucose-3-¹⁴C, it was necessary to start with the three-carbon compound D-glycerose (used as its 2,3-O-isopropylidene derivative) and extend the carbon chain to six carbon atoms, by three successive, addition reactions. Carbon-¹⁴-labeled

cyanide was used only in the first of these additions. The use of carriers in each step would have resulted in such dilution of radioactivity as to have made the final product useless. Consequently, we employed large-scale paper-chromatography for separations at the tetronic and pentonic levels. In order to ascertain optimal conditions for the first cyanohydrin reaction, the rate and completeness of addition of cyanide in various buffers were determined. The results, given below, show that the most rapid and complete reaction with cyanide occurred in the presence of sodium bicarbonate and sodium carbonate.

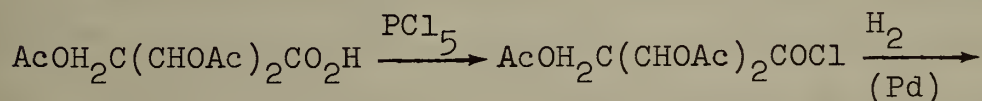
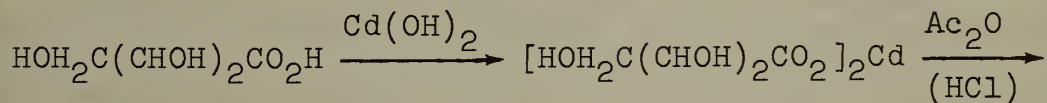
Table 1. Completeness of cyanide addition to 2,3-O-isopropylidene-D-glycerose.

Buffer	Time (days)				
	3	5	7	10	12
	%	%	%	%	%
NaHSO ₃	67.3	69.6	70.7	74.1	75.0
NaHCO ₃	78.6	88.7	94.6	97.7	98.9
Na ₂ CO ₃	72.7	82.7	88.5	93.7	95.6
NaOAc } HOAc }	67.3	73.2	75.5	77.9	79.1

Because of the absence of the stabilizing pyranoid ring in the tetroses, tetronic lactones cannot be reduced to tetroses by the methods successfully employed for reducing pentonic and higher lactones. Thus, both sodium amalgam reduction and catalytic hydrogenation of an erythronic lactone lead to the fully reduced product, erythritol [12,13].

However, a modified Rosenmund reduction [14], as first used by Cook and Major on fully acetylated aldonyl chlorides [15], appeared to provide a possible semimicro method for preparing D-erythrose-1-¹⁴C. The method had already been applied by Glattfeld and Kribben [13] to the macro-reduction of DL-erythronic acid (to give an over-all yield of DL-erythrose of less than 4%). Subsequently, Ladenburg et al. [16] improved the yields of acetylated aldonic acids by use of the cadmium salt of the acid for the acetylation. For the semimicro preparation of tri-O-acetyl-D-erythronyl chloride, it seemed probable that phosphorus pentachloride, used by Cook and Major, would be more convenient than thionyl chloride, employed by Glattfeld and Kribben. In pilot experiments on non-labeled material, one-millimole quantities of cadmium D-erythronate were freeze-dried, acetylated with a mixture of acetic anhydride and hydrogen chloride, converted into tri-O-acetyl-D-erythronyl chloride with phosphorus pentachloride, and reduced by the Rosenmund procedure to tri-O-acetyl-aldehydo-D-erythrose. The over-all yield, as determined by titration, was 90%.

The reactions are as follows:



The chromatographic separation of epimers at the 4-carbon stage proved to be complicated by the formation of condensation products. Consequently, the tetronic acid mixture was converted into the two D-tetroses-1-¹⁴C, and thence into a mixture of the four D-pentonic-2-¹⁴C acids. After lactonization and paper chromatography, this gave, as one fraction, a mixture of 2-¹⁴C-labeled D-arabinonic and D-xylonic lactones. This mixture of lactones was reduced to a mixture of the corresponding sugars, and D-arabinose-2-¹⁴C was separated chromatographically; this sugar was then used for the third cyanohydrin synthesis. The entire reaction scheme, given in Figure 2 yielded not only the desired D-glucose-3-¹⁴C but also numerous by-products of great value for the study of reaction mechanisms, for the determination of isotope effects, and for use as starting compounds in the preparation of other labeled carbohydrates.

B. Synthesis of Tritium-labeled Carbohydrates

1. General Methods

The versatility of a ¹⁴C-labeled compound in chemical and biological research can be greatly extended by the simultaneous use of the tritium-labeled compound; various double-label techniques are then possible. Implemented by liquid scintillation spectrometry, double-label methods have wide application in biological studies, in evaluation of isotope effects, and in determination of reaction mechanisms. For these reasons, the Section has developed (a) methods for the

	Radioactivity (millicuries)
2,3-O-Isopropylidene-D-glycerose	
↓ cyanohydrin synthesis (Na^{14}CN)	132
Epimeric D-tetronic cyanohydrins	
↓ hydrolysis	
↓ neutralization of acids with $\text{Cd}(\text{OH})_2$	
Cadmium D-erythronate-1- ^{14}C and D-threonate-1- ^{14}C	116
↓ Tri-O-acetyl-D-erythranyl-1- ^{14}C and D-threonyl-1- ^{14}C chlorides	
↓ Rosenmund reduction	
Tri-O-acetyl-aldehydo-D-erythrose-1- ^{14}C and D-threose-1- ^{14}C	95
↓ cyanohydrin synthesis (NaCN)	
↓ hydrolysis	
Four D-pentonic acids	
Aliquot Taken for Remaining Steps	68 (71%)
↓ lactonization of acids	
↓ chromatographic separation	
{ A. D-Lyxono-1,4-lactone-2- ^{14}C (held on paper)	-
B. D-Ribono-1,4-lactone-2- ^{14}C (held on paper)	-
C. D-Arabinono-1,4-lactone-2- ^{14}C	19.0
D-Xylono-1,4-lactone-2- ^{14}C	
↓ reduction of band C (NaHg_x)	
chromatographic separation	
D-Xylose-2- ^{14}C (held on paper)	-
D-Arabinose-2- ^{14}C	7.0
↓ cyanohydrin synthesis (NaCN)	
Epimeric D-hexonic cyanohydrins	
↓ hydrolysis, and separation of acids by use of carriers	
D-Mannono-1,4-lactone-3- ^{14}C	3.0
Potassium D-gluconate-3- ^{14}C	3.6
D-Glucono-1,5-lactone-3- ^{14}C	
↓ reduction (NaHg_x)	
D-Glucose-3- ^{14}C	2.8

Figure 2. Synthesis of D-glucose-3- ^{14}C .

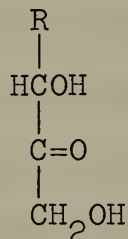
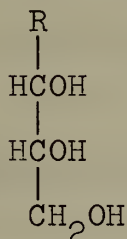
synthesis of tritium-labeled as well as carbon-14-labeled carbohydrates, and (b) techniques for using double-labeled compounds.

Carbohydrates position-labeled with tritium must be synthesized by methods entirely different from those already described. In general, reduction processes (carried out with tritiated reagents, such as the tritiated metal hydrides) have proved to be the most convenient and practicable for position-labeling. Thus, reduction of an aldehyde group then yields a primary alcohol having a carbon-bound tritium atom. Reduction of a keto group likewise introduces tritium, and simultaneously creates a new asymmetric center; the two tritium-labeled diastereoisomers resulting can usually be separated by chromatography. Reduction of aldonic lactones produces aldoses-1-t and alditols-1-t; the proportions depend on the experimental conditions.

We have also used other reduction methods, such as (a) catalytic tritiation and (b) reduction of lactones with sodium amalgam in tritiated water. Method (a) requires a closed system for circulating tritium gas, but yields high-activity products; method (b) necessitates the use of excessive amounts of radioactivity in the form of tritiated water, because the tritium introduced comes from the solvent. However, method (b) is convenient for preparing low-activity aldoses-1-t. Some synthetic procedures developed in this laboratory are given in references 17 to 20, inclusive.

2. Preparation of Intermediates

Because reduction of a >C=O group to a $\text{>C}^{\text{T}}\text{<OH}$ group is an effective method for introducing carbon-bound tritium into a carbohydrate molecule, we investigated carbohydrate intermediates possessing the former group. Some of these intermediates are commercially available [e.g., 2-keto- and 5-keto-D-gluconic acid (D-arabino-2-hexulosonic acid and D-xylo-5-hexulosonic acid), and the common uronic acids (D-glucuronic, D-mannuronic, and D-galacturonic acids)]. Other needed carbonyl compounds can be obtained only by laborious chemical syntheses, involving oxidation of a carbohydrate derivative in which all but one >CHOH group is protected by O-substitution. A third group of carbonyl intermediates may be obtained by certain stereospecific, bacterial oxidations. Thus, Acetobacter suboxydans accomplishes the following oxidation:



Agrobacterium tumefaciens oxidizes C-3 of many D-glucopyranosyl and D-galactopyranosyl derivatives.

3. 3-t-Labeled Carbohydrates

We have applied all of the described methods (for the preparation of intermediates and reduction thereof) in the

synthesis of a number of aldoses and alditols labeled at C-1, C-2, and C-6. Work is now in progress on the preparation of certain important 3-t-labeled carbohydrates.

Through the kindness of Professor M. Bernaerts, Brussels, we have received samples of 3-keto-lactose and 3-keto-maltobionic acid. By means of a culture of Agrobacterium tumefaciens, we have oxidized several disaccharides and aldobionic acids, and, by chromatography, have shown the presence of the corresponding 3-keto compounds. By use of various tritiated reducing reagents, these 3-keto compounds are being reduced either differentially (keto group only) or completely. The products will be chromatographically separated either before or after hydrolysis of the disaccharide linkages. The resulting 3-t-labeled carbohydrates, particularly sucrose, lactose, D-glucose, and D-galactose will have many important applications. There are innumerable analytical and technological possibilities for the use of the tritium-labeled carbohydrates; these will undoubtedly be developed when the materials become available.

C. Preparation of Carbon-14- and Tritium-labeled Carbohydrates for the Scientific Public

After development, the described synthetic methods have been used for preparing sufficient supplies of radioactive sugars to furnish needed quantities to other laboratories. Each preparation requires careful handling through a complex series of processes, and final proof of a high degree of purity, usually by chromatographic methods.

As radioassay methods have grown more sophisticated, research workers have become concerned with smaller and smaller amounts of radiochemical impurities. Since, on storage, decomposition of radioactive organic compounds can occur by self-radiation, we periodically monitor our supplies by chromatography and, when it is needed, supply information to other workers concerning purity and methods of synthesis and analysis. This project fills an urgent need on the part of medical and biological research workers. Although the simpler radioactive carbohydrates are now made commercially, the Bureau remains an important source in this country for difficultly synthesized radioactive carbohydrates.

D. Isotope Dilution and Double-label Methods of Analysis

The analysis of organic materials is greatly facilitated by the use of carbon-14- and tritium-labeled compounds, of which the Organic Chemistry Section has a unique and valuable collection. The usefulness of radioisotopes arises in large measure from the ease and sensitivity of measurement of radioactivity. One of the most useful analytical techniques, isotope dilution, permits the analysis of mixtures that are otherwise extremely difficult to evaluate. Thus, an aliquot of a mixture of labeled products can be added to a known quantity of a (non-labeled) suspected constituent, and the total activity of the carrier (purified by recrystallization or chromatography) indicates the amount of the labeled constituent in the aliquot. The method can be used whenever pure compounds are available.

The technique is even more effective in the so-called double-label method. In this, an aliquot of an unknown mixture, labeled with radioisotope A, is added to a suspected constituent having a known activity of radioisotope B. Amounts of the doubly labeled compound too small for gravimetric procedures can then be isolated by chromatography or by addition of non-labeled carrier (because such addition does not change the ratio of A to B). The activity of A in the aliquot (in the form of the given compound) is then calculated from this ratio and the amount of radioisotope B that was added.

We have developed a combination of the two techniques just described which permits the kinetic study of isotope effects, or differences in the rates of reaction of labeled and non-labeled molecules. In this analytical technique, both isotopes are present in the compound before reaction; one is situated at or near the reaction center, and the other is remote from it. When an isotope is directly involved in a rate-determining step of a reaction, a relatively large (primary) isotope effect occurs. An isotopic atom remote from the reaction center has little or no effect on the reaction rate. If an isotope effect exists in the reaction of a doubly labeled compound, the ratio of the two isotopes in both the residual reactant and the product changes progressively as the reaction proceeds, and the isotope effect can be determined from this change. At various stages of the reaction, non-labeled carriers of either the reactant or the

product are added to aliquots of the reaction mixture, and the ratio of isotopes is determined in the doubly labeled compound. The method is particularly suitable for use with carbon-14 and tritium.

E. Determination of Kinetic Isotope-effects

The isotope effect is usually expressed as $\underline{k^*}/\underline{k}$, in which $\underline{k^*}$ and \underline{k} are, respectively, the rate constants for the labeled and non-labeled forms of the reactant molecule. For a reaction that is first order (or pseudo first-order) with respect to the reactant, the value of $\underline{k^*}/\underline{k}$ can be calculated by the equations of Stevens and Attree [21] as modified by Ropp [22]:

$$\underline{k^*}/\underline{k} = 1 + [\log \underline{r}' / \log (1 - \underline{f})] \quad (1)$$

$$\underline{k^*}/\underline{k} = \log(1 - \underline{rf}) / \log(1 - \underline{f}) \quad (2)$$

In these equations, \underline{f} is the fraction of starting material that has reacted. When the equations are applied to radioactive compounds, \underline{r} is the ratio of the molar specific activity of the accumulated product to that of the initial reactant; and \underline{r}' is the ratio of the molar specific activity of the residual reactant to that of the initial reactant.

(The value for the molar specific activity of the accumulated product of 100% reaction may be used instead of that for the initial reactant.)

Determination of \underline{f} , \underline{r} , and \underline{r}' by conventional methods is difficult, because the product and the residual reactant must be separated and rigorously purified. However, the task has been greatly simplified by the simultaneous use of two radioisotopes [23]. If \underline{p}^0 is the ratio of the functional to the

reference isotope in the initial reactant, \underline{p} the ratio in the residual reactant, and \underline{p}' the ratio in the product, then $\underline{r} = \underline{p}'/\underline{p}^0, \underline{r}' = \underline{p}/\underline{p}^0$, and equations 1 and 2 become, respectively:

$$\underline{k^*}/\underline{k} = 1 + [\log(\underline{p}/\underline{p}^0)\log(1 - \underline{f})] \quad (3)$$

$$\text{and } \underline{k^*}/\underline{k} = \log(1 - \underline{f}\underline{p}'/\underline{p}^0)/\log(1 - \underline{f}) \quad (4)$$

When the reaction is complete, \underline{p}' becomes equal to \underline{p}^0 (provided that the functional isotope is retained in the product and that there are no side reactions).

By means of the analytical method outlined, values for $\underline{k^*}/\underline{k}$ for carbon-14- and tritium-labeled compounds involved in the following reactions and processes were measured and reported in the publications cited: Oxidation of labeled D-mannitols and D-glucitols with Acetobacter suboxydans [24, 25]; recrystallization of D-glucose-1-t (single label only) [26]; oxidation of D-glucose-1-t and D-glucose-1-¹⁴C with iodine [23]; recrystallization of D-mannose-1-t phenylhydrazine [27]; oxidation of aldoses-1-t with bromine [28]; oxidation of aldehydes and aldoses-1-t with sodium chlorite [29].

On the basis of the isotope effects determined, a unique mechanism was proposed for the oxidation of aldehydes and aldoses with chlorite ion. The mechanism accounts, for the first time, for the unusual stoichiometric relationships in this reaction.

The work on the oxidation of aldoses-1-t with bromine (a currently controversial subject) showed clearly that various amounts of the slowly oxidized α anomers mutarotate to the β

anomers before oxidation, and that the relative amounts of the α anomers oxidized directly and indirectly depend on the conformational stability of the pyranose form.

An analytical method was also developed for kinetically determining a tritium isotope-effect, without the use of double labels, for reactions in which water-t is formed from a nonvolatile reactant [30]. In this method, the equation of Ropp is employed, and f is estimated from the amount of reagent consumed or by other convenient means. The solution containing the products of partial reaction and the residual reactant is diluted with water to a known volume. A portion of this solution is freeze-dried, and the total activity of the water-t (the product of partial reaction) is calculated. The ratio of the activity of the water-t to the activity of the initial reactant equals rf. Substituting the values of f and rf in equation (2) gives $\underline{k^*}/\underline{k}$. The precision of the method is illustrated in Table 2. Results obtained by the double-label method, given in Table 3, show that the agreement is good, considering that the values from the water-t method are based on the early part of the reaction period, whereas those from the double-label method are based on the latter part.

Table 2. Isotope effects in oxidation of aldoses-l-t with iodine in alkaline solution at 25 °C.

Aldose- <u>l-t</u>	Fraction of aldose oxi- dized	Radioac- tivity in original reactant	Radioac- tivity of water- <u>t</u> formed	Isotope effect
	<u>f</u>	<u>μc</u>	<u>μc</u>	<u>k*/k</u>
<u>L</u> -Arabinose	0.100	6.05	0.092	0.15
	.200	6.30	.194	.14
	.300	5.64	.262	.13
			Avg.	0.14
<u>D</u> -Xylose	.165	15.90	.347	.12
	.200	13.78	.511	.17
	.303	13.83	.756	.16
			Avg.	0.15
<u>D</u> -Glucose	.100	7.60	.120	.15
	.200	7.43	.252	.15
	.300	7.56	.409	.16
			Avg.	0.15
<u>D</u> -Mannose	.107	9.82	.143	.13
	.198	7.55	.214	.13
	.313	7.47	.334	.12
			Avg.	0.13
<u>D</u> -Galactose	.101	6.26	.082	.12
	.199	6.35	.186	.13
	.317	6.42	.340	.14
			Avg.	0.13
<u>D</u> -Talose	.100	10.86	.194	.17
	.200	11.26	.340	.14
	.300	10.09	.515	.15
			Avg.	0.15
<u>L</u> -Rhamnose monohydrate	.100	16.71	.239	.14
	.200	11.28	.330	.13
	.300	12.40	.504	.12
			Avg.	0.13

Table 3. Determination of isotope effects in the oxidation of aldoses-l-t with iodine in alkaline solution by the double-label technique.

Fraction of aldose oxidized	Ratio of functional to reference isotope in carrier mixture	Isotope effect
<u>f</u>	<u>p</u>	<u>k*/k</u>
<u>D-Glucose-6-¹⁴C-l-t</u>		
0.0	9.12	----
.761	30.89	0.15
.785	30.66	.21
.839	43.05	.15
.882	52.17	.18
		<hr/>
		Avg. 0.17
<u>D-Mannose-2-¹⁴C-l-t</u>		
0.0	7.72	----
.726	23.43	.14
.756	26.39	.13
		<hr/>
		Avg. 0.14

F. Special Apparatus and Techniques

In the foregoing sections, we have omitted description of the techniques employed in the preparation and use of radioactive carbohydrates. In this section, we describe general procedures, apparatus, and techniques which have been developed (or modified) for the synthesis or analysis of carbon-14 or tritium-labeled compounds. The techniques are chiefly modifications of procedures developed in many laboratories [31]. Much of the synthetic work on labeled carbohydrates is carried out on a semimicro scale in test tubes or round-bottomed flasks having standard-tapered joints (19/38 or 24/40). The tubes are conveniently made from blank, standard-tapered, outer joints; hollow stoppers sufficiently long to accomodate a spatula are made from the inner joints (Figure 3).

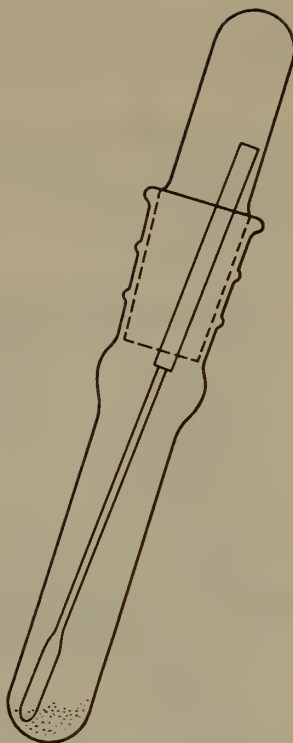


Figure 3. Standard-tapered test tube with radioactive sugar.

1. Transfer of Liquids

Several methods are used for the transfer or removal of radioactive solutions. Formerly, disposable pipets were drawn from Pyrex-glass tubing (O.D. 7 mm) and fitted with rubber bulbs of 1- or 2-ml capacity; this type of pipet is now commercially available.

A special filtration pipet, made in the Section, is often used for removing mother liquor from crystals in a test tube (Figure 4). To make the pipets, high-melting glass (Vycor) is crushed in a mortar, and successively sieved through screens of 40, 60, and 100 mesh per inch. Pyrex-glass tubing (O.D. 8 mm) is drawn out, and so cut that the



Figure 4. Sintered-glass filter pipet.

narrow opening is about 1 or 2 mm in diameter. The narrow end of the tube is then tamped in the sieved glass collected by either the 60-mesh or the 100-mesh sieve, so that about 2 to 5 mm of the end of the tube is filled with glass particles of the desired size; the tamped end is then slightly sintered in a flame. From a series of pipets so prepared, only those that have a satisfactory flow-rate under vacuum are selected; in use, the sintered-glass pipet, equipped with a rubber bulb, serves as a small, fritted-glass filter. Usually, radioactive compounds are not removed from the test tube in which they are crystallized; instead, the mother liquor is separated by means of a sintered-glass pipet, the crystals are appropriately washed, and the washings are similarly removed.

It is often convenient to transfer a liquid from one flask or test tube to another; the simple apparatus of Figure 5 is effective for such transfers. The flask is rinsed in the same manner in which it is drained. Formerly, narrow, glass tubing was used for connecting the two containers; however, narrow, flexible-plastic tubing, which is now available, obviates breakage and the need for several sizes of glass siphons.

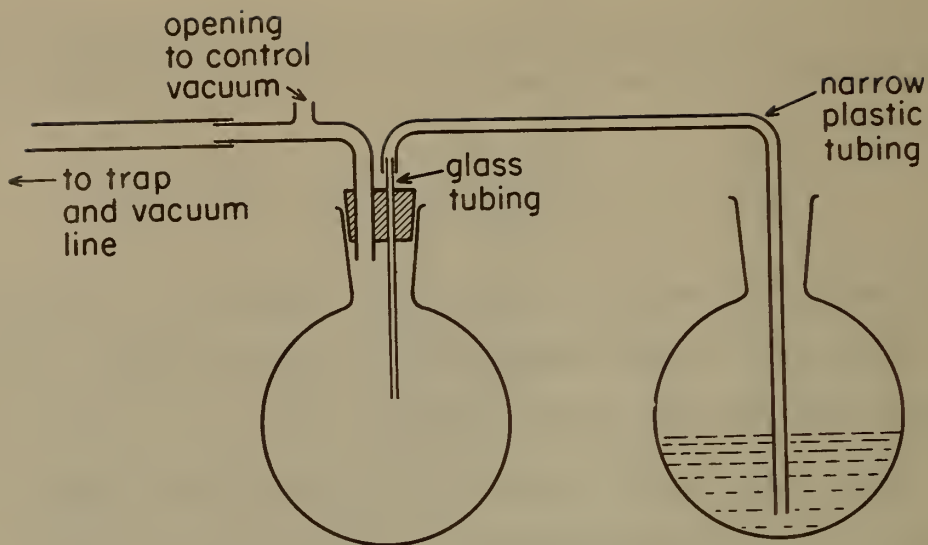


Figure 5. Apparatus for transfer of liquids.

2. Filtration

For filtering radioactive solutions, we devised the glass funnel of Figure 6, which is used in a variety of sizes, the larger and wider ones containing a perforated porcelain or fritted-glass plate. The opening of a small funnel is usually closed with a plug of glass wool or a glass "nail" made by flattening the end of a narrow glass rod; this is covered with a layer of paper pulp (tamped down) and, if decolorization is necessary, with a small amount of a decolorizing carbon (provided that the solution to be filtered does not have a high specific radioactivity). If more than a few milliliters of solution are to be filtered, a transfer siphon (shown in the sketch) is fitted to the funnel, permitting the transfer of a radioactive solution without loss.

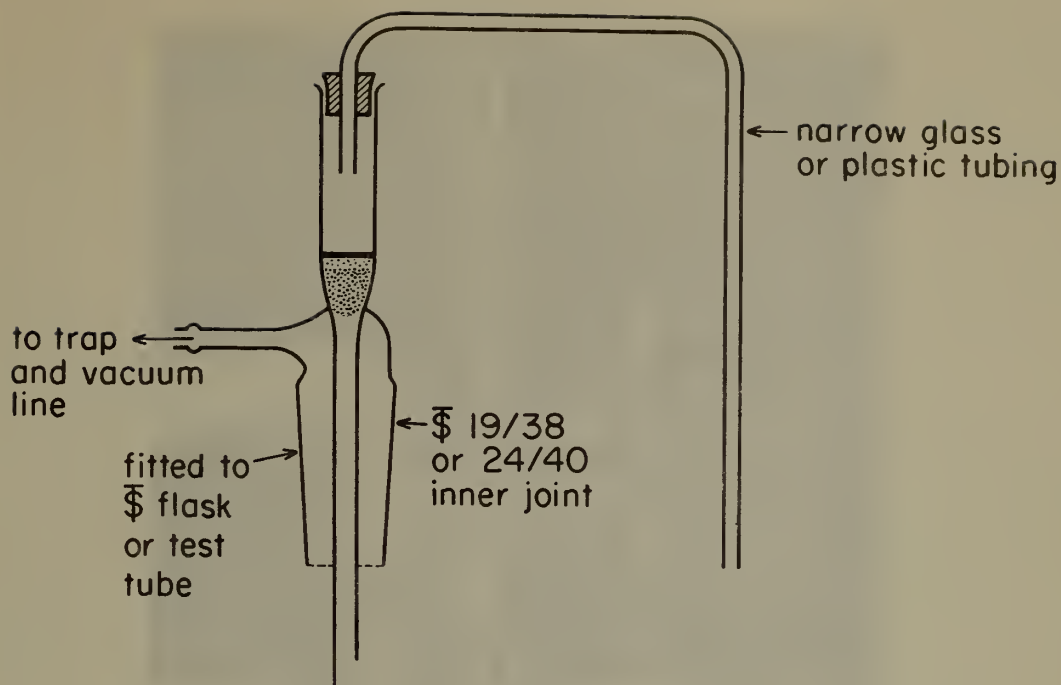


Figure 6. Filtration apparatus.

Figure 7 shows apparatus designed for filtering solutions of compounds in a closed system. At the start of the process, flask C contains the solvent, and flask A (initially in the lower position) contains the solid to be dissolved. Under vacuum, solvent is distilled from flask C to flask A, and the solid is dissolved. Flasks A and B are then rotated 180 degrees, flask B is cooled, and the solution filters through the fritted-glass disk into flask B. By cooling the top of flask A (with powdered, solid carbon dioxide supported on the flask by a cork ring) and slightly warming flask B, solvent condenses on the upper wall of flask A, thereby rinsing it. This solvent returns to flask B when flask B is again cooled.

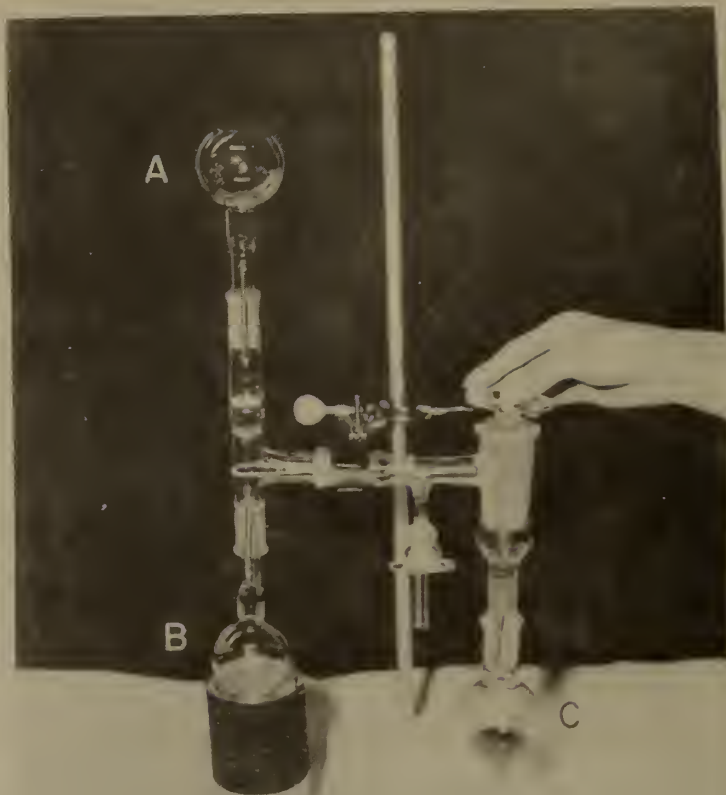


Figure 7. Apparatus for filtration in a closed system.

The solution in flask B may be freeze-dried (see following section), or the compound may be brought to crystallization by evaporating part of the solvent on a rotary vacuum still or by adding a poor solvent.

We designed the apparatus of Figure 8 for use in evaluating tritium isotope-effects in reactions in which water-t is evolved, as, for example, in the water-t method described on page 15 of this report.

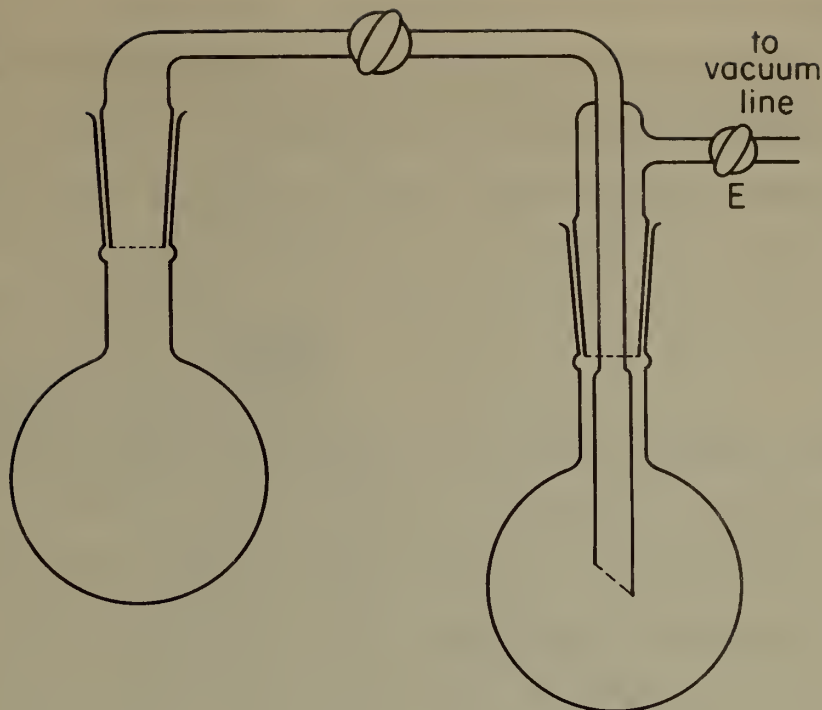


Figure 8. Distillation apparatus for determining water-t. vacuum line.

3. Removal of Solvents

Solvent removal has been one of the most serious problems in the development of methods for synthesizing radioactive carbohydrates. For concentration of the relatively large volumes of solution often obtained as the eluant from columns of ion-exchange resins, we have used a rotary vacuum still of the conventional type; however, each distillate is tested for radioactivity (that occasionally may be mechanically carried into the distillate as spray). If radioactive, the distillate is separately re-evaporated, and, when reduced to a small volume, is transferred to the principal radioactive residue.

Water, acetic acid, and p-dioxane are frequently

removed from solutions of radioactive materials by freeze-drying. A solution is frozen in a round-bottomed flask, and the solvent is removed by sublimation. Figure 9 shows the use of an "activity trap" (A) so designed that solid

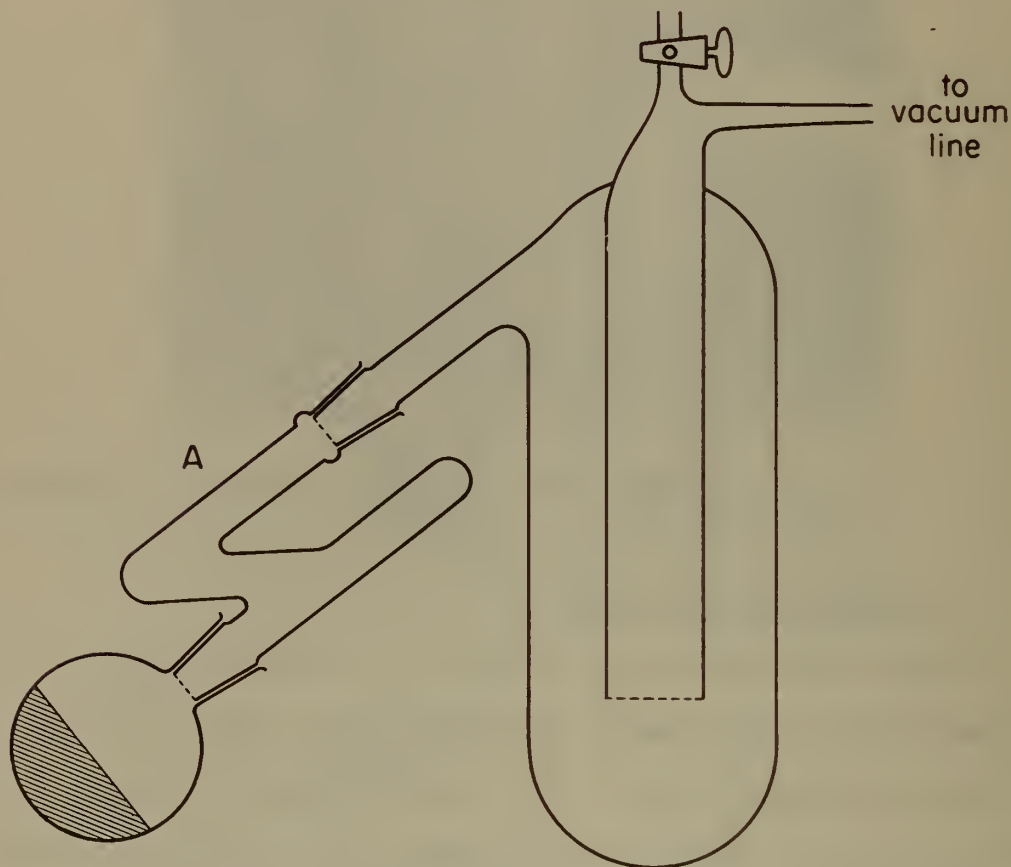


Figure 9. Trap for preventing entrainment losses in freeze-drying.

radioactive residues (which, if fluffy, may tend to puff out of the flask) may be readily reclaimed. The contents of the solvent trap (which is cooled in a mixture of solid carbon dioxide and an organic solvent) are tested for radioactivity before they are discarded.

To concentrate liquids in test tubes, we have used a commercially available, multiple-outlet, vacuum still (modified for use with standard-tapered test tubes) that rapidly swirls the contents of the tubes in a bath at constant temperature. The apparatus is used with a cold trap and a vacuum pump.

4. Reduction of Aldonic Lactones by Sodium Amalgam

In slightly acid solutions, lactones of aldonic acids are reduced by sodium amalgam to the corresponding sugars. Because the reaction of the amalgam with water produces sodium hydroxide, it has been customary to add acid, usually sulfuric, from time to time as the solution approaches neutrality. However, for the reduction of radioactive aldonic lactones with sodium amalgam on a semimicro scale and in a closed system, it was necessary to devise new procedures and apparatus. Instead of the step-wise addition of sulfuric acid, we use an excess of (slightly soluble) sodium hydrogen oxalate. The reductions are conducted in a heavy-walled, standard-tapered glass tube that has a side arm for adding amalgam (Figure 10); the tube is fitted with a stainless-steel stopper having a greaseless bearing that accommodates a stainless-steel stirrer. In use, the reduction tube is kept in an ice bath (Figure 11). The sodium amalgam used in the reduction is prepared in the form of pellets (Figure 12) by pouring molten amalgam into a column of mineral oil [32].

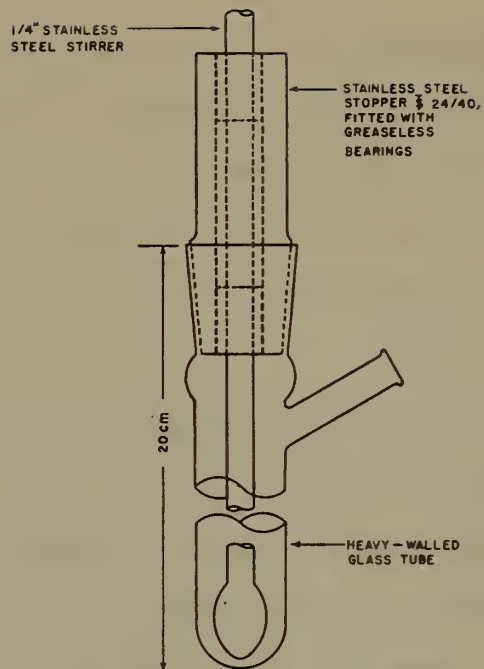


Figure 10. Sodium amalgam reduction apparatus.



Figure 11. Apparatus for reduction of aldonic lactones.

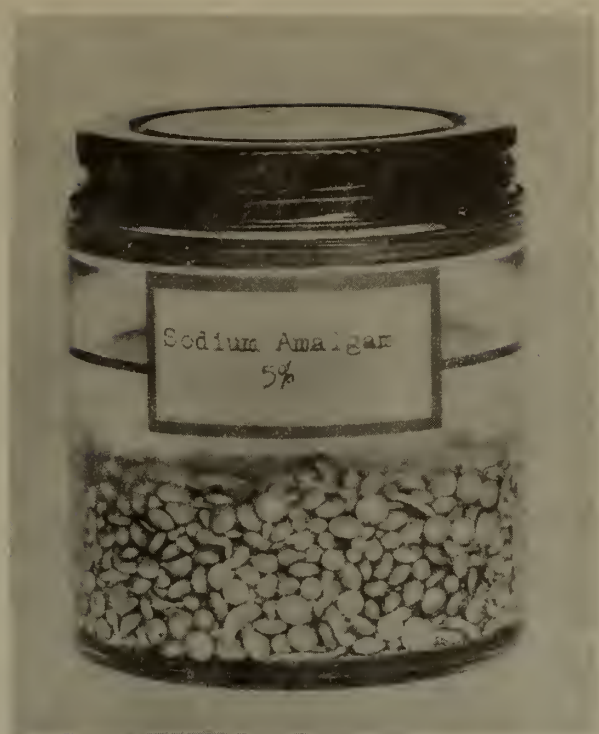


Figure 12. Sodium amalgam in the form of pellets.

Prior to the reduction of a ^{14}C -labeled lactone, the amount of sodium amalgam required for the maximal yield of the corresponding sugar is determined by reducing one-millimole samples of the non-labeled lactone with various quantities of sodium amalgam in the presence of excess (solid) sodium hydrogen oxalate. In a typical preparation of a radioactive sugar, one millimole of the ^{14}C -labeled aldonic lactone and 2 g of sodium hydrogen oxalate are placed in the reduction tube (Figure 11). Twenty milliliters of ice water is then added, and, after the stirrer has been started, 4.6 g of (5%) sodium amalgam pellets are introduced into the tube through the side arm.

The mixture is vigorously stirred in the ice bath for about an hour. The reduction tube is then removed from the ice bath, mercury is pipetted from the mixture, and the solution is neutralized by the addition of sodium hydroxide solution (sufficient to produce a faint, permanent pink color with phenolphthalein indicator); the color is then discharged by the dropwise addition of a solution of oxalic acid. Five volumes of methanol are added, and the crystalline salts are removed by filtration, washed with methanol, and discarded. The combined filtrate and washings are concentrated under reduced pressure to a thin sirup, to which four volumes of methanol are added; the small crop of salts thus precipitated is removed and washed as before. The filtrate is freed from methanol under reduced pressure, and an aqueous solution of the product is passed through a column of mixed cation- and anion-exchange resins (about 20 ml). The resin is washed with water until the radioactivity of the last part of the effluent is negligible. The combined eluant is tested for ionic impurities by use of a commercial conductivity-meter of the type used for testing the purity of distilled water. The salt-free solution is concentrated under reduced pressure, and freeze-dried (Figure 9). The residue, dissolved in a few drops of water, is transferred to a tared test-tube (Figure 3). The solution is diluted with two volumes of methanol, nucleated with the crystalline sugar, and treated with

sufficient 2-propanol to produce incipient turbidity. Additional 2-propanol is added as crystallization proceeds. Finally, the mother liquor is removed by means of a capillary filter-pipet (Figure 4), and the crystals are washed with a mixture of methanol and 2-propanol. The compound is recrystallized, usually with filtration of the solution (Figure 6), until it is chromatographically pure.

5. Chromatography.

We have used paper chromatography extensively in synthesizing and testing both carbon-14- and tritium-labeled compounds. Radioautographs of chromatograms of carbon-14-labeled compounds are useful for detecting impurities (Figure 13), or following the course of a reaction. For certain difficult separations or purifications, large-scale paper chromatography has been used for preparative purposes. Bands containing carbon-14-labeled



Figure 13. Radioautograph of chromatogram of impure ^{14}C -labeled sugar.

compounds, precisely located by radioautographs, are cut out of the paper, and eluted.

Chromatograms of tritium-labeled compounds, because of the weaker radiation, do not produce satisfactory radioautographs. However, by use of a commercial chromatogram-scanner, it is possible to ascertain the purity of tritium-labeled compounds (Figure 14), and even



Figure 14. Scan of chromatogram of a pure tritium-labeled sugar.

to determine the location of bands of tritium-labeled compounds on a large-scale, preparative chromatogram. These techniques will be discussed in more detail in a forthcoming paper.

6. Techniques for the Assay of Carbon-14 and Tritium

Utilization of carbon-14 and tritium as research tools requires simple and precise methods of analysis. The method

of choice depends on the equipment at hand and the substance to be analyzed. In prior publications from this Section, techniques have been described for determining carbon-14 and tritium by assaying samples in films [33, 34] and in solution [4] with a 2- π , windowless, gas-flow, proportional counter. The methods require relatively simple equipment, but they are laborious.

At present, most of our measurements are made with a dual-channel, liquid scintillation counter which greatly simplifies measurements of radioactivity. With this instrument, assays of carbon-14 and tritium may be made separately, or simultaneously with double-labeled compounds, without changing the setting of the instrument. This flexibility in the use of the dual-channel instrument is achieved in the conventional manner [35], by critically setting the pulse-height discriminators of channel 1 for monitoring tritium, those of channel 2 for monitoring carbon-14, and the voltage of the photomultiplier tubes for optimum, over-all monitoring.*

* For the simultaneous assay of carbon-14 and tritium in a liquid-scintillation counter, Okita and co-workers [36] described three procedures, which they designated as (a) the screening method, (b) the simultaneous-equation method, and (c) the discriminator-ratio method. The procedure outlined here is essentially their method (b). Under the conditions described, the method has the advantage that, without changes in setting, it permits counting of either isotope alone, or of the two in the same sample, with fairly high efficiency.

In order to obtain the best settings for the pulse-height discriminators, plots are made of the counting efficiencies, at various pulse-heights, of both carbon-14 and tritium in the scintillator solution of choice. The optimal setting is taken as that point on the graph at which the highest contrast-ratio (ratio of the counts occurring in one channel to those occurring in the other) is obtained for both isotopes with minimal loss of counting efficiency for channel 1 (tritium). For selecting the voltage of the photomultiplier tubes, integral, discrimination-bias curves (counting efficiency versus high voltage) are plotted for each isotope. From these curves, obtained with discriminator settings of 40 to 500 v (for channel 1) and 300 to 1000 v (for channel 2), for the instrument at hand, an optimal setting of 1225 v was found for counting carbon-14 and tritium in the p-dioxane-containing scintillator solution described below. The value for the high voltage is the optimal neither for carbon-14 nor for tritium, but is a compromise that permits (a) satisfactory counting of either isotope separately, and (b) simultaneous counting of the two isotopes in a mixture.

We have found scintillator solutions prepared from p-dioxane or toluene as described by Hayes [37] to be highly satisfactory; because of considerations of solubility we ordinarily use the former solvent. The p-dioxane

scintillator solution contains 7 g of PPO^* , 0.3 g of dimethyl-POPOP^{**}, and 100 g of naphthalene per liter; the toluene scintillator solution contains 5 g of PPO^* and 0.3 g of dimethyl-POPOP^{**} per liter. By use of 0.1-ml aliquots of aqueous, radioactive solutions in 10-ml quantities of the p-dioxane scintillator solution, and with the settings given above, we have found the following counting efficiencies: in channel 1, about 26% for tritium and 25% for carbon-14; and in channel 2, about 3.7% for tritium and 72% for carbon-14.

The radioactivity of each isotope in a mixture containing both carbon-14 and tritium (T) may be expressed by the following equations [36]:

$$\frac{{}^{14}\text{C}}{\text{dps}} = \frac{N_1 h_2 - N_2 h_1}{c_1 h_2 - c_2 h_1}, \text{ and} \quad (5)$$

$$\frac{\text{T}}{\text{dps}} = \frac{N_1 c_2 - N_2 c_1}{h_1 c_2 - h_2 c_1} \quad (6)$$

where N_1 = net counts per second of channel 1, N_2 = net counts per second of channel 2, h_1 = T efficiency of channel 1, h_2 = T efficiency of channel 2, c_1 = $\frac{{}^{14}\text{C}}$ efficiency of channel 1, c_2 = $\frac{{}^{14}\text{C}}$ efficiency of channel 2, and dps = disintegrations per second. After the four counting

* 2,5-diphenyloxazole.

** 2,2'-p-phenylenebis(4-methyl-5-phenyloxazole).

efficiencies have been determined, equations 5 and 6 can be simplified (by combining constants) to the following convenient forms:

$$\underline{\underline{^{14}\text{C}}}_{\text{dps}} = \text{AN}_2 - \text{BN}_1 \quad (7)$$

$$\underline{\underline{\text{T}}}_{\text{dps}} = \text{CN}_1 - \text{DN}_2 \quad (8)$$

The values of the combined constants A, B, C, and D apply to a given set of conditions only.

This method of counting double-labeled compounds requires careful determination of counting efficiencies. The best results are obtained when the $\underline{\underline{\text{T}}}/\underline{\underline{^{14}\text{C}}}$ ratio lies between 20:1 and 1:1.

In order to check the accuracy and precision of the double-label counting procedure, we prepared and assayed a series of solutions containing (in each solution) known amounts of $\underline{\underline{\text{D}}}\text{-glucose-}\underline{\underline{1-}}\underline{\underline{^{14}\text{C}}}$ and $\underline{\underline{\text{D}}}\text{-glucose-}\underline{\underline{1-t}}$. In each analysis, 0.100 ml of an aqueous solution was counted in 10 ml of the p-dioxane scintillator solution. Each sample was counted five times, for 100 seconds each time. The results are reported in Table 4.

Table 4. Determination of tritium:carbon-14 ratios with a dual-channel liquid-scintillation counter.

Composition of sample			Counts/second			Found		Difference ^a (%)		
\overline{T}	$\frac{^{14}C}{dps}$	$\frac{T}{^{14}C}$ ratio	Channel 1 N_1	Channel 2 N_2	\overline{T} dps	$\frac{^{14}C}{dps}$	$\frac{T}{^{14}C}$ ratio	\overline{T}	$\frac{^{14}C}{dps}$	$\frac{T}{^{14}C}$
3022	152.8	19.78	814.69	217.37	2991	148.7	20.11	-1.0	-2.7	+1.7
2296	155.4	14.77	627.81	195.02	2267	154.8	14.64	-1.3	-0.4	-0.9
1915	155.3	12.33	530.97	179.21	1898	151.7	12.51	-0.9	-2.3	+1.5
1507	153.4	9.82	426.51	163.98	1497	151.1	9.91	-0.7	-1.5	+0.9
1183	152.5	7.75	345.15	152.67	1184	151.5	7.82	+0.1	-0.7	+0.9
1558	310.5	5.02	474.17	277.47	1533	307.0	4.99	-1.6	-1.1	-0.6
706	721.1	0.98	364.80	556.55	706	737.0	0.96	0.0	+2.2	-2.0
131	676.4	0.193	197.35	488.92	123	673.0	0.183	(-6.1	-0.5	-5.2)

Standardization

1439	----	374.12	53.10	$h_1 = 26.00; h_2 = 3.69$
----	310.4	76.61	223.98	$c_1 = 24.68; c_2 = 72.15$

^aPercent found minus 100

2. SUGARS IN SOLUTION

(H. S. Isbell, H. L. Frush, R. Schaffer,
C. W. R. Wade and R. A. Peterson)

For over 100 years, the complex and manifold reactions of the sugars in alkaline solution have fascinated organic chemists and engaged their attention. Notwithstanding painstaking and extensive investigations, knowledge of the interconversion and degradation reactions of sugars in alkaline solution is still incomplete.

In a monumental work, Nef and his students spent many years in separating and identifying the products of the alkaline degradation of sugars, but obtained very few quantitative data [38]. Modern, tracer methods of analysis are far more powerful than the classical methods, and the reaction mixtures can now be readily analyzed. However, the problem is complicated by the existence of several competing reactions, some of which are reversible and some not. Determination of the equilibrium proportions of the products of the reversible reactions, and the relative rates for all of the reactions, would be of great value to workers in theoretical organic chemistry.

Our Section is in a particularly favorable position to obtain much of the desired information because we have high competence in the preparation, purification, and analysis of carbohydrates, as well as an extensive collection of

carbon-14- and tritium-labeled sugars that are useful for following the course of the reactions by tracer techniques.

In developing the program, we have studied the main processes involved in the transformations; these include (a) mutarotation reactions, (b) enolizations, (c) reversible condensation and cleavage reactions, and (d) rearrangements of the saccharinic acid type.

A. Mutarotation Reactions

When dissolved in water, reducing sugars undergo certain reversible, isomerization reactions which result in changes in optical rotation known as mutarotation. The rationalization of mutarotation reactions is an intriguing and important problem in carbohydrate chemistry.

The mutarotation process is known to involve the reversible interconversion of several modifications of the sugar [39]. For each sugar, this results in an equilibrium between two pyranose forms, two furanose forms, an open-chain form, and solvated and ionic modifications of these. The mutarotations of D-galactose, D-talose, D-ribose, D-glycero-D-ido-heptose, and certain other sugars give clear evidence for two types of reaction, namely, α - β pyranose interconversions and pyranose-furanose interconversions [40]. The mutarotations of D-glucose, D-xylose, lactose, and certain other sugars arise almost entirely from reversible α - β pyranose interconversions. The mutarotation of D-fructose,

however, arises in large measure from a pyranose-furanose interconversion [41].

The α - β pyranose interconversions (reactions of type I), have been investigated thoroughly, but relatively little work has been done on pyranose-furanose interconversions (reactions of type II). Isbell and Pigman [41] showed that the two types of reaction differ markedly in their heats of reaction, activation energies, rates of reaction, and sensitivity to acid-base catalysts. It has been tacitly assumed that α - β pyranose and pyranose-furanose interconversions take place through a common, open-chain intermediate. Other mechanisms are possible, especially a change in configuration without ring cleavage, or a change in the size of the ring by an intramolecular, opposite-face mechanism. In view of these possibilities, it seemed desirable to study both types of mutarotation, in water and in deuterium oxide. Prior to the present work, others had studied the mutarotation of D-glucose and certain other sugars by isotope effects [42], and had obtained important information on the reaction mechanisms, but no pyranose-furanose interconversion had been so investigated. Therefore, we undertook a study of isotope effects in the mutarotation of D-glucose, a typical α - β pyranose interconversion, and the mutarotation of D-fructose, a typical pyranose-furanose interconversion. The work has now been reported in a Ph.D. thesis by a member of the staff (C.W.R. Wade) [43].

The ultimate objective of the work was to elucidate the mechanisms for pyranose-furanose interconversions and to show how these reactions differ from α - β pyranose interconversions. The research plan included the following tasks: (a) Development of convenient methods for preparation and study of crystalline deuterated sugars; (b) determination of highly accurate data on the rates of mutarotation for certain sugars in H_2O , in the presence of acid and base catalysts, and for the same sugars in D_2O , in the presence of deuterated acids and bases; (c) determination of the Arrhenius heats of activation for the two types of reaction in H_2O and in D_2O ; (d) comparison of the catalytic effects of acid and base catalysts on the two types of reaction in H_2O and in D_2O ; (e) determination of the relative rates of mutarotation in H_2O and in D_2O (isotope effects) for the acid-, base-, and water-catalyzed reactions of types I and II; and (f) formulation of satisfactory mechanisms for the two types of mutarotation reaction.

In accordance with this plan, methods were successfully developed for the preparation and purification of the O-deuterium analogs of α -D-glucopyranose, α -D-xylopyranose, and α -D-fructopyranose. The rates of mutarotation of the O-hydrogenated sugars in H_2O and of the O-deuterated sugars in D_2O , were determined in the presence of sulfuric acid (0.015 M, 0.0025 M, and 0.00125 M), potassium acid phthalate (0.001 M), and o-nitrophenol (0.01 M), at 3.9° and 20.0°C.

The rates of mutarotation of α -D-glucopyranose in H_2O and D_2O in the presence of 0.0006 M sodium hydroxide were also determined. The rate constants were used to calculate the activation energies, acid-base catalytic effects, and isotope effects ($\underline{k}/\underline{k}^*$). Catalytic-rate equations were calculated to show the relative effectiveness of the catalytic pairs $\text{H}_3\text{O}^+/\text{D}_3\text{O}^+$, OH^-/OD^- , and $\text{H}_2\text{O}/\text{D}_2\text{O}$. In all cases, for a given pair of catalysts and at a given temperature, "normal" isotope effects ($\underline{k}/\underline{k}^* > 1$) were found for the rates of mutarotation of each sugar. The results were examined and interpreted as functions of α - β pyranose or type I interconversions (for D-glucose) and of pyranose-furanose or type II interconversions (for D-fructose).

The activation energies, catalytic effects, and rate constants clearly show parallel differences in type I and type II reactions in both solvent systems. However, both the measured and the calculated (catalytic) isotope effects show that three distinct but similar mechanisms are concurrently operative for both types of reaction. In strongly acid solutions, both α - β pyranose and pyranose-furanose interconversions occur by rapid, pre-equilibrium, proton transfers, followed by the rate-determining step. In slightly acid solutions, proton transfers occur in the rate-determining step. In basic solutions, pH measurements and isotope effects show that the sugar loses a proton to the base in a rapid step

and that the formation of the sugar anion precedes the rate-determining step. Thus, in highly acid solution, the general acid-catalyzed mechanism predominates; in slightly acid solution, the water-catalyzed mechanism prevails; and, in alkaline solution, the base-catalyzed mechanism is most important.

The results support reaction mechanisms advanced by Isbell and Frush in an earlier publication [44]. In each instance, the open-chain form of the sugar is the intermediate in the mutarotation reaction. Although present in small proportions, the open-chain intermediates give rise to reactions characteristic of aldehydic and ketonic compounds. Some of these will be considered next.

B. Enolization Reactions

1. Successive, Reversible Processes

In alkaline solution, the open-chain form of the sugar in the equilibrium mixture undergoes enolization. By this process, an aldose yields a 1,2-enediol, and a ketose yields both a 1,2- and a 2,3-enediol. According to Nef [38], reversion of a 1,2-enediol gives two epimeric aldoses plus the corresponding ketose, and reversion of the 2,3-enediol gives two ketoses having the carbonyl group at C-2 plus two ketoses having the carbonyl group at C-3. Enolization of the resulting two 3-ketoses yields a 2,3-enediol plus two 3,4-enediols. Reversal of the process, beginning with each of the

3,4-enediols, gives (hypothetically) two ketoses having the carbonyl group at C-3, plus two having the carbonyl group at C-4. Presumably, by this type of reaction, all of the aldohexoses and ketohexoses possible are formed from a single hexose.

The early work of Lobry de Bruyn and Alberda van Ekenstein [45] provided clear evidence for the formation of the 1,2- and 2,3-enediols; however, the existence of the 3,4- and 4,5-enediols of hexoses remained uncertain. Formation of D,L-sorbose by alkaline rearrangement of D-glucose and of D-fructose indicated possible extensive isomerization [46], but the authors suggested that the L-sorbose may arise by fragmentation and recombination rather than by successive enolization and de-enolization. Later, by use of D-glucose-1-¹⁴C Sowden and Thompson [47] established that D,L-sorbose is formed almost exclusively by the enolization—de-enolization mechanism (which must include formation of the 3,4- and 4,5-enediols), and that fragmentation and recombination play only a minor role in the isomerization. The existence of 3,4-enediols was recently confirmed [48] by R. Schaffer of our staff, who prepared crystalline β -D-manno-3-heptulose monohydrate and found that it rearranges in alkaline solution, via a 2,3-enediol, to D-gluco-heptulose, and, via a 3,4-enediol, to a transitory, epimeric 3-ketose. This intermediate yields a 2,3-enediol and, ultimately, D-altro-heptulose plus D-allo-

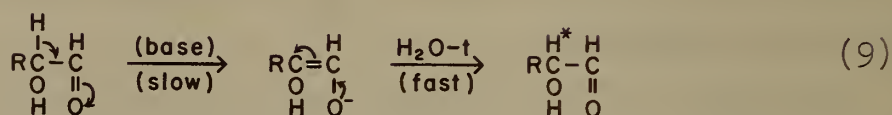
heptulose. Thus, Nef's idea of the existence of all possible enediols is confirmed.

Ordinarily, an equilibrium state is not reached in these interconversion reactions. Hence, the proportions of the isomeric sugars in the reaction mixtures depend on the extent of the reaction. In the early stages of the reaction, the composition of the reaction mixture depends on the rates of formation of the various sugars. In the final stage of the reaction (pseudo-equilibrium state) the proportions of the sugars present in the mixture depend on their relative thermodynamic stability. To understand the behavior of sugars in alkaline solutions, we need to know, for each sugar, the rate of enolization, the relative rates of conversion of the enediols into the several sugars, and the relative thermodynamic stability of each sugar in the pseudo-equilibrium mixtures.

2. Measurement of Rates of Primary Enolization

Heretofore, quantitative measurements of the rates of enolization have not been attempted because the system is so complicated. However, we have recently developed a method for measuring the rates of enolization of the sugars under a variety of conditions. The method involves treating the sugar under any desired condition with a base catalyst in the presence of tritiated water. After a short reaction time, the mixture is acidified. This introduces carbon-bonded

tritium into the molecule (see equation 9). Assay of the



reaction mixture for tritium, after removal of excess tritiated water and oxygen-bonded tritium, provides a measure of the enediol formed. By acidifying the reaction mixture when only a small fraction of the sugar has been converted into enediol, one obtains a measure of the rate of the primary enolization. By this method, we have measured the rate of enolization of D-glucose in the presence of various catalysts. The results are now being prepared for publication, and similar measurements are being made for other sugars.

3. Measurement of Rates of Interconversion of Sugars

Prior to the present work, Sowden and Schaffer [49] had applied tracer methods to the study of the alkaline rearrangement of D-glucose, D-mannose, and D-fructose in water and in deuterium oxide. They fully confirmed the mechanism for the interconversion of the three sugars through the 1,2-enediol, and showed the practicability of tracer methods for obtaining detailed information on the reactions.

The following method has now been developed for studying the interconversion reactions: A sample of the ^{14}C -labeled sugar (usually 0.1 millimole) is treated with a base catalyst under controlled conditions. After the desired reaction

period has elapsed, the reaction mixture is treated with a cation-exchange resin to remove the base. The solution is then passed through an anion-exchange resin to remove the saccharinic acids. The carbon-14 retained on the resin is a measure of the saccharinic acids formed. The deionized solution is divided into aliquots, and the labeled products are measured by the isotope-dilution technique, using non-labeled carriers.

We have now completed measurements on the rearrangements of D-glucose, D-fructose, and D-mannose at various temperatures, in 0.036-N calcium hydroxide, in 0.04-N sodium hydroxide, in dry pyridine, and in aqueous pyridine. Interconversion of the sugars at 5°C is surprisingly slow. After nine months, 85 to 90% of the starting sugar remained intact. Under the conditions used, there is no appreciable difference in the action of calcium hydroxide and sodium hydroxide. We found that, in the early stages of the reactions, (1) D-glucose gives D-mannose and D-fructose in nearly equal proportions; (2) D-mannose gives much more D-fructose than does D-glucose; and (3) D-fructose gives more D-mannose than does D-glucose.

A study of the interconversion reactions of D-galactose, D-talose, D-sorbose (D-xylo-hexulose), and D-tagatose (D-lyxo-hexulose) is also in progress. The work entails very careful purification of the labeled sugars used as tracers and of the sugars used as carriers. We plan to study, ultimately, the

interconversions of all of the hexoses and of as many pentoses and heptoses as possible. Measurements made in the early stages of the reactions give information on the relative rates of formation of the various sugars one from another. The measurements supplement the measurements of the rates of enolization made by use of tritiated water (see Section B.2). Measurements of the composition of mixtures obtained after long reaction-periods are being used to estimate the relative stabilities of the isomeric hexoses. This subject will be considered next.

4. Thermodynamic Stability of Isomeric Hexoses

In an equilibrium system, Gibbs free-energy differences can be calculated from the equation $\underline{G}^{\circ} = -RT \ln K$. Because some of the reactions of sugars in alkaline solution are not reversible, accurate equilibrium constants cannot be obtained. Approximate values can be estimated from the proportions of the various sugars in pseudo-equilibrium states. By approaching the pseudo-equilibrium state from each of the constituent sugars, it may be shown that the equilibrium constants lie between certain values. We have estimated equilibrium constants from the ratios of D-mannose to D-glucose and D-fructose to D-glucose in two reaction systems — one, aqueous 0.04-N sodium hydroxide at 35°C and the other, 9-percent aqueous pyridine at 120°C. With aqueous pyridine at 120°C, the free-energy difference in calories for conversion of D-glucose into D-mannose was found to be of the order of +1000, and, for

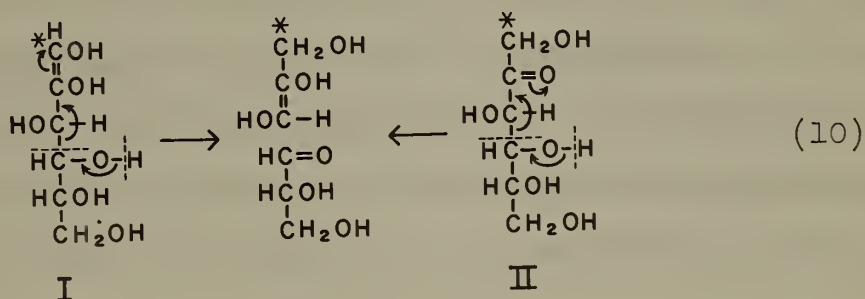
D-glucose into D-fructose, of the order of +300 calories per mole. The study is being continued in order to obtain more-accurate values.

C. Reversible Condensation and Cleavage Reactions

In alkaline solution, sugars establish equilibrium states consisting of furanose and pyranose modifications, together with small proportions of carbonyl and enolic forms. We have already considered sugar transformations, in alkaline solution, that do not involve a change in the carbon skeleton of the sugar. However, certain rearrangements occur with alteration of the carbon chain. Some of these take place by cleavage of the carbon chain followed by resynthesis by aldol condensation.

1. Cleavage and Resynthesis of Hexoses

Tracer methods provide a means for evaluating the alteration in the carbon chain. In a rearrangement starting with a sugar that is position-labeled with carbon-14, the extent of cleavage and resynthesis can be estimated from changes in the distribution of carbon-14 in the substrate and in the product. Thus, cleavage of a hexose-1-¹⁴C yields a 1-labeled triose-enediol and non-labeled D-glyceraldehyde as shown in equation 10.



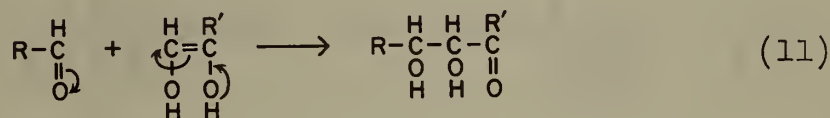
The cleavage may start with either the 1,2-enediol (I) or the free ketose (II), and may proceed by the electron shifts depicted in the formulas. Presumably, the keto path is the more important [50]. The nascent, labeled triose-enediol equilibrates with dihydroxyacetone (1,3-dihydroxy-2-propanone) and becomes labeled at both C-1 and C-3. Except for the label, the enediol is the same as the enediol of D-glyceraldehyde. Hence, both fragments become labeled at C-1 and C-3, and the hexulose formed by aldol condensation of the labeled fragments would be labeled at carbon atoms 1,3,4, and 6. To provide information on possible cleavage and resynthesis reactions, we have degraded some of the sugars obtained by rearrangement of the labeled sugars in the tracer studies already described. Our results show that the cleavage and resynthesis reaction in the preparation of D-fructose-1-¹⁴C from D-glucose-1-¹⁴C by the action of 0.04-N alkali does not exceed 2% of the total reaction.

Presumably, under highly alkaline conditions, the cleavage and resynthesis reactions reach pseudo-equilibrium states. It might be expected that repetitive aldol condensations would give compounds having long carbon-chains. But, surprisingly, the pseudo-equilibrium mixtures obtained by alkaline rearrangement of lower sugars do not contain appreciable proportions of sugars having more than six carbon atoms. Inasmuch as the composition of the mixture at equilibrium is determined by thermodynamic stability, it may be concluded

that sugars having more than six carbon atoms are less stable than the lower sugars.

2. Formation of Straight-chain Ketoses and Branched-chain Aldoses by the Aldol Condensation

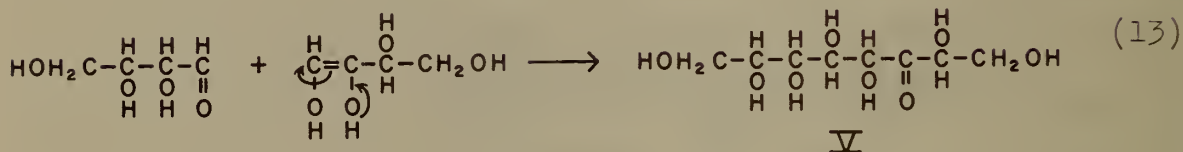
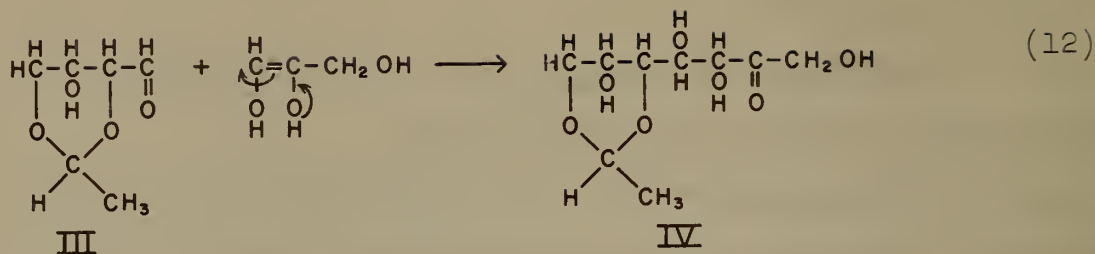
In an aldol condensation, one reactant must be in the carbonyl form and the other in the enolic form, as shown in equation 11.



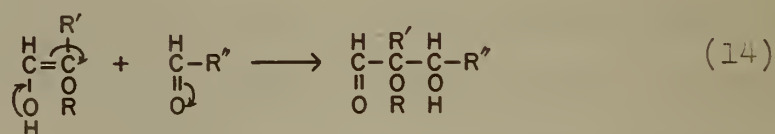
The two components of the addition reaction may be (a) tautomeric forms of the same compound, or (b) different compounds. Depending on the nature of the reactants, it is possible to obtain either linear ketoses or branched-chain aldoses. Our results show that, when the hydroxyl group at C-2 of the enediol is unsubstituted, linear ketoses are formed. In the unsubstituted enediol, hyperconjugation of the R' group at C-2 enhances the nucleophilic properties of C-1, and hence promotes substitution at this point.

Thus, Schaffer and Isbell found that 2,4-O-ethylidene-D-erythrose (III) and dihydroxyacetone (presumably in the enolic form) yield a straight-chain 5,7-O-ethylideneheptulose (IV) [51]; and Schaffer and Cohen found that, by self-condensation, D-erythrose, in alkaline solution, yields a straight-chain 3-octulose (V) [52]. Presumably, the reactions take place as

shown in equations 12 and 13.



When the hydroxyl group at C-2 of the enediol is substituted, an electron shift occurs from the hydroxyl group at C-1 to carbon atom 2. This shift increases the nucleophilic properties of C-2, with the result that the addition takes place between this carbon atom (instead of C-1) and C-1 of the aldehyde, to yield a branched-chain aldose. The mechanism is depicted in equation 14.

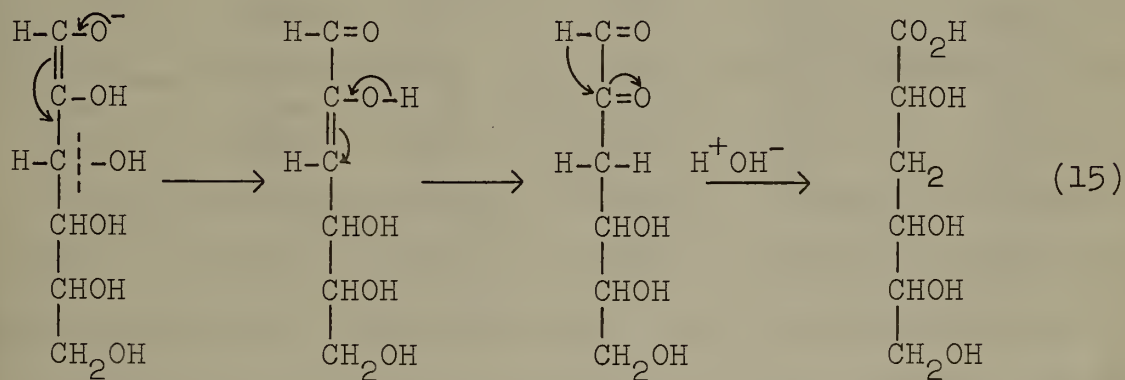


As discussed in Section 3.A of this report, Schaffer and Isbell [53,54] found that branched-chain aldoses are formed in high yield by condensation of sugar derivatives in which the hydroxyl of C-2 is blocked by substituent groups. Formation of the branched-chain compounds is unusual, and has not heretofore been explained.

D. Rearrangements of the Saccharinic Acid Type

In addition to the reversible aldol condensations just discussed, essentially non-reversible, saccharinic acid rearrangements take place in alkaline solutions of sugars. As pointed out by Nef [38], saccharinic acid formation takes place through isomerization of the sugar to an α -dicarbonyl compound, followed by a benzilic acid type of rearrangement of the latter, with hydration. However, Nef's concept of the mode of isomerization of the original sugar to the intermediate α -carbonyl compound did not prove sound. But, in 1944, an acceptable course for the initial isomerization was developed by Isbell [55]. In the revised mechanism, the α -dicarbonyl intermediates for production of the various types of saccharinic acid arise from enolization followed by β -elimination of a hydroxyl group.

Thus, formation of metasaccharinic acid was depicted as in equation 15.



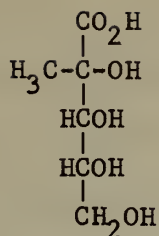
1,2-Enediol

Metasaccharinic
acid

Similar mechanisms were formulated for the production of saccharinic acids from 2,3- and 3,4-enediols, and these have been largely confirmed [50].

However, tracer studies reveal that, in some instances, saccharinic acids may be formed by two or more paths. Thus, in the formation of " α "-D-glucosaccharinic acid (VI) from D-mannose-1-¹⁴C, Sowden, Blair, and Kuenne [56] found 39% of the carbon-14 at the methyl carbon atom (the expected position) and 57% at the tertiary carbon atom. As pointed out by Kenner and Richardson [57], carbon-14 at the tertiary carbon atom could arise from fragmentation and resynthesis of the sugar, followed by rearrangement of the 1,3-labeled hexose. However, Sowden, Blair, and Kuenne noted that this process, if operating alone, could account for only 50% of the isotope at the tertiary carbon atom; this proportion would be lessened by direct isomerization of the hexose-1-¹⁴C.

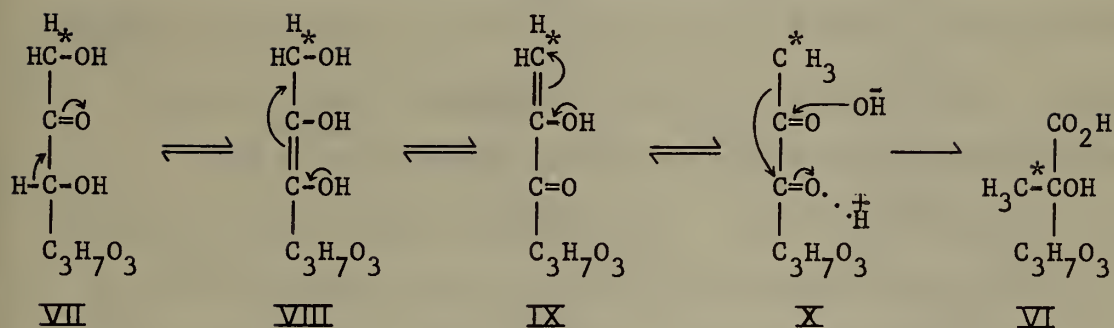
Lemieux suggested [58] that the unexpected result might arise from two concurrent reactions, one by way of the Nef-Isbell mechanism, and the other by way of an internal-return mechanism in which D-mannose-1-¹⁴C (XI) is converted into D-glucose-2-¹⁴C (XII), which then rearranges, through D-fructose-2-¹⁴C (XIII) and the conventional mechanism, to " α "-D-glucosaccharinic acid (See Figure 15). According to this proposal, Sowden, Blair, and Kuenne's result would require, as an intermediate, D-fructose labeled at C-1 and C-2 in the ratio of 39 to 57, respectively.



" α "-D-Glucosaccharinic Acid
(2-C-Methyl-D-ribo-pentonic Acid)

VI

Nef-Isbell Mechanism



Lemieux Mechanism

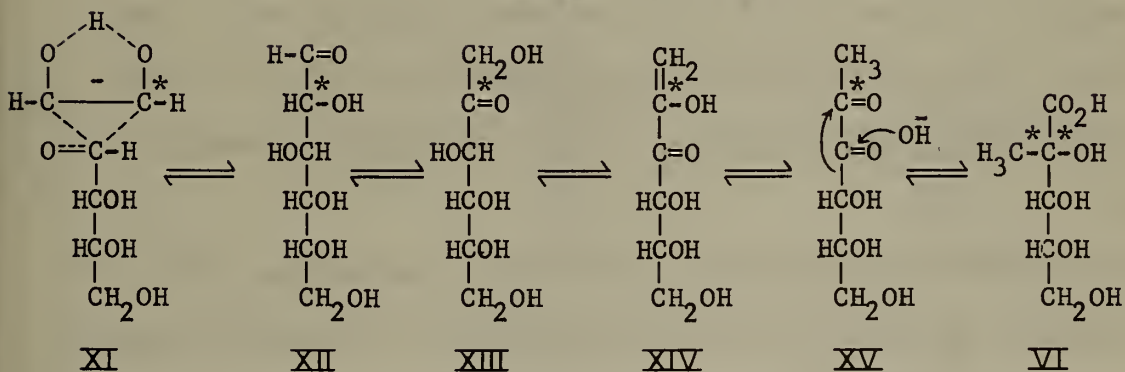


Figure 15. Prior mechanisms for the formation of " α "-D-glucosaccharinic acid.

We have now separated and analyzed the labeled-D-fructose formed from D-mannose-1-¹⁴C under conditions similar to those used for preparing "α"-D-glucosaccharinic acid. Alkaline degradation of the labeled D-fructose to the next lower aldonic acid (D-arabinonic acid) showed 98% of the carbon-14 to be at C-1. Hence, the rearrangement cannot take place by the mechanism suggested by Lemieux. The lack of carbon-14 in the labeled D-fructose at positions other than C-1 also shows that Kenner and Richard's proposed fragmentation of D-mannose and recombination cannot play a major role in the rearrangement.

Because none of the proposed mechanisms fully account for the experimental results, we have formulated a new mechanism (see Figure 16). It differs from others in that the "α"-glucosaccharinic acid labeled at the tertiary carbon atom is derived from C-3 of the parent sugar, instead of from C-2, as in the Lemieux mechanism. The mechanism effects an interchange of C-1 and C-3 of the metasaccharinic acid. We believe that the new mechanism, in conjunction with the Nef-Isbell mechanism, will account for the supposedly anomalous results.

The Nef-Isbell mechanism, the Lemieux mechanism, and the newly proposed mechanism involve a benzylic acid rearrangement of the same α-diketone. The diketones for the three mechanisms are produced by different paths and are labeled in different positions. Presumably, in each instance, the benzylic acid rearrangement may occur in either of two ways, depending on

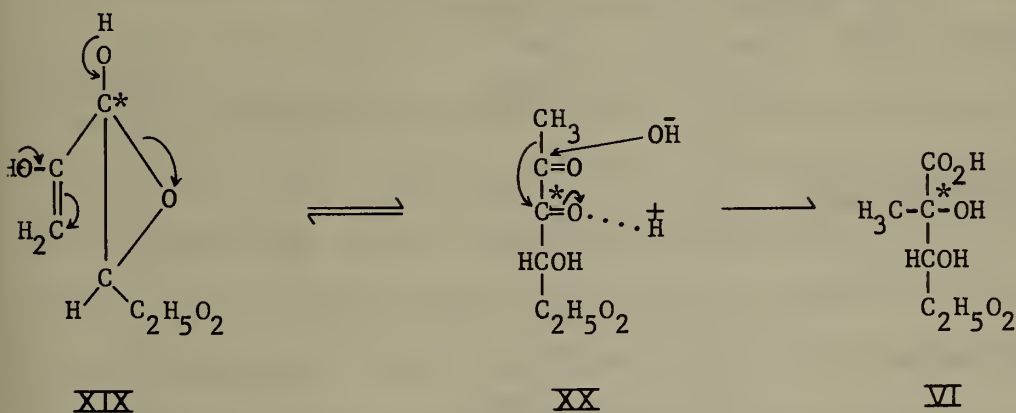
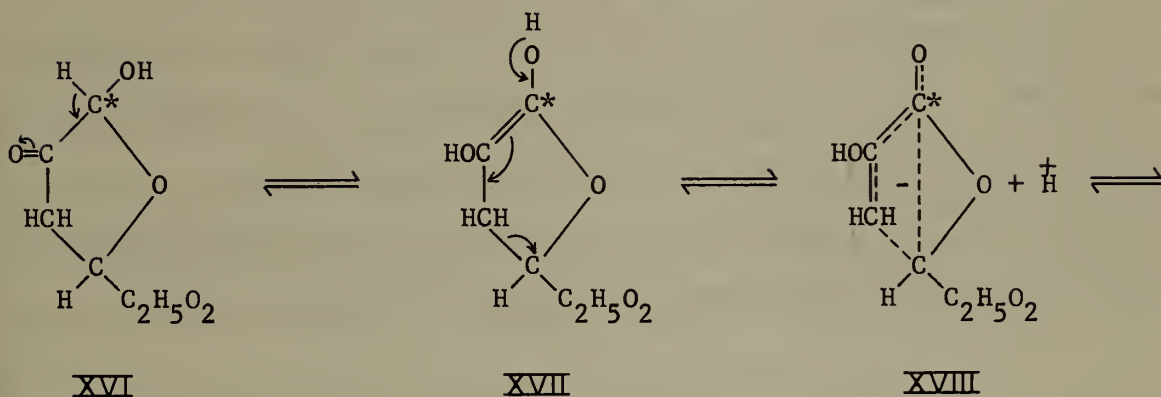
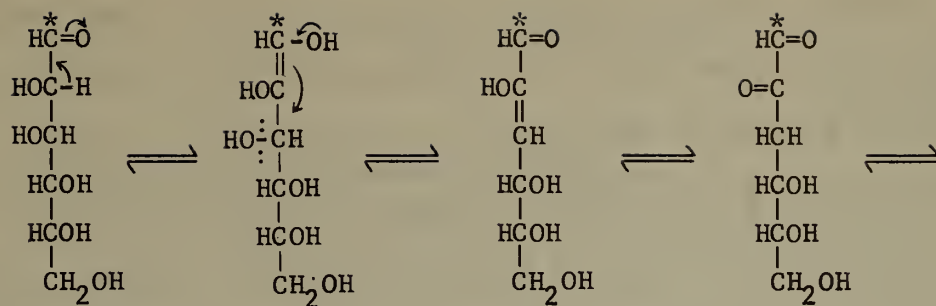


Figure 16. Proposed mechanism for the formation of "α"-D-glucosaccharinic acid.

which of the two carbonyl groups is attacked by the hydroxyl ion. To account for the presence of carbon-14 at the tertiary carbon atom of the product from D-mannose-1-¹⁴C, with the new mechanism the attack of the hydroxyl ion must be at C-2, causing migration of the methyl group to C-3; with the Lemieux mechanism, the attack must be at C-3, causing migration of the C₃H₇O₃ group to C-2. Table 5 shows the positions of C-1, C-2, and C-3 of the parent D-mannose in the "α"-D-glucosaccharinic acid that would be formed by the three mechanisms. To test the proposed mechanism experimentally, we have rearranged samples of D-mannose-1-¹⁴C and D-mannose-3-¹⁴C, and are now determining the distribution of carbon-14 in the resulting "α"-D-glucosaccharinic acid.

Table 5. Migration of carbon atoms in the formation of "α"-D-glucosaccharinic acid.

	<u>If CH₃ migrates</u>	<u>If C₃H₇O₃ migrates</u>
Nef-Isbell Mechanism	C-1 in CH ₃	C-1 in CH ₃
	C-2 in CO ₂ H	C-2 in R ₃ COH
	C-3 in R ₃ COH	C-3 in CO ₂ H
Lemieux Mechanism	C-1 in CO ₂ H	C-1 in R ₃ COH
	C-2 in CH ₃	C-2 in CH ₃
	C-3 in R ₃ COH	C-3 in CO ₂ H
Proposed Mechanism	C-1 in R ₃ COH	C-1 in CO ₂ H
	C-2 in CO ₂ H	C-2 in R ₃ COH
	C-3 in CH ₃	C-3 in CH ₃

3. ALDOL CONDENSATIONS

(R. Schaffer)

A. Syntheses via Aldol Condensations

The sustained interest that carbohydrates have commanded because of their immense economic value and the ever-widening recognition of their biochemical importance and variety has resulted in the characterization of complete families of these substances. This unique assemblage of linear and cyclic stereoisomers has been put to use in modern organic chemistry as model compounds in studies of reaction mechanisms involving stereochemical transformations and the subtleties of molecular shape (conformation). However, some of these compounds are not easily obtained from the syntheses through which they are known; also, there are some, as yet unknown, that should be made, for they would favorably complement those currently available. The possibilities of devising easier access to some of the known compounds and to desirable new ones are twin objectives of the stereochemistry project. We have chosen to accomplish these objectives by use of seemingly complex syntheses which have been neglected in the past because they presented the troublesome prospects that their use would lead to a multiplicity of products and to severely increased stereochemical problems; however, such obstacles may today be overcome by employing the effective separation techniques and powerful analytical instrumentation that have been recently developed.

Our interest in these complex syntheses began while undertaking a detailed investigation of the structure of a dialdopentose derivative. In studying it, we were able to attribute the unusual properties of the compound to the presence of a hemiacetal form of a 1,3-dioxane ring that joined two molecules together [59]. However, this stabilizing feature vanished in the presence of alkali, which, we found, catalyzed an unexpected aldol condensation between two molecules of the dialdose, yielding a 4-C-formyldialdo-nonose, probably the most complex monosaccharide known. Its discovery indicated that aldol condensations, although not typical reactions of carbohydrates, might usefully be secured with other suitably selected derivatives [53, 54]. This was proved possible with the synthesis of a 2-C-(1,2-dihydroxyethyl)-D-glucose [60] from D-ethylidene-D-erythrose. (The method of preparation of D-erythrose presented in this paper has been recommended above any other proposed [61].) In addition, the potentiality of aldol reactions of aldose derivatives was further demonstrated by utilizing two different reactants for the preparation (in excellent yield) of a 4-C-(hydroxymethyl)pentose [62]. Although of importance in themselves as branched-chain sugars (related, for example, to streptose, a carbohydrate component of streptomycin), the new substances proved to be useful source-materials from which exceedingly valuable compounds could be derived; thus, from the 4-C-(hydroxymethyl)pentose,

the enantiomorph of the rare, natural sugar D-apiose was readily prepared [62]; and D-manno-3-heptulose, the first known example of a 3-ketose, was obtained from the 2-(1,2-dihydroxyethyl)-D-glucose [60]. This extraordinary ketose had a structure thought to have only transitory existence as an intermediate in isomerization reactions. Its properties gave convincing evidence for previously proposed enediol mechanisms [48], and settled a controversial attempt to ascribe a 3-heptulose structure to a carbohydrate isolated from a biological source [63]. In addition to the 3-heptulose, we were able to make available a second crystalline, unsubstituted 3-ketose that was discovered to be an aldol-condensation product of D-erythrose [52]. The readiness with which D-erythrose undergoes transformation to D-gluco-L-glycero-3-octulose makes probable to us that it will be observed in biological systems.

With the aid of a former summer employee, Mr. Eugene Barron, evidence of the formation of a third 3-ketose has been obtained in an aldol condensation of D-threose. It is hoped that work with this new substance can soon be resumed. We have learned, by private communication, that our experiments are stimulating interest in other laboratories in the chemistry of these compounds.

We have been able, also, to devise a simple aldol synthesis for higher ketoses having their keto group at

carbon atom 2. The problem of separating isomeric products was encountered in this process, but was simply overcome through the use of chromatography, as these products were previously known compounds [51]. Their nature and composition were found to be altered when the same reactants in unsubstituted form were allowed to enter the reaction. In this way, the previously difficultly obtainable D-allo-heptulose, which was not detected in the aldol synthesis with substituted reactants, could be isolated in a useful yield [64].

B. Cyclopentitols

At present, we are concerned with the synthesis and characterization of cyclopentane derivatives having a functional group on each carbon atom. Surprisingly little is known of pentasubstituted cyclopentanes. Such products would be the 5-carbon analogs of the cyclitols, which function as the model compounds for stereochemical studies on cyclohexanes. The cyclopentane derivatives may be expected to serve similarly for cyclopentane chemistry, and they should be particularly valuable in shedding light on cyclopentane conformations. Our cyclopentane derivatives are being prepared from a new compound which is unusual in having two hemiacetal groups in one ring of its structure. In a series of reactions, it is converted into certain novel monoamino-tetrahydroxycyclopentane derivatives. Assignment of configuration to each of the asymmetric carbon atoms of the

products is now under investigation.

Our success in elucidating structural features of the new compounds with which we are concerned is related to a combination of reliance on the classical approach of organic chemistry and utilization of the remarkable information that analytical instrumentation can provide.

4. MOLECULAR STRUCTURE

(H. S. Isbell, A. J. Fatiadi, E. J. McDonald)

A. Cyclic Polyhydroxy Ketones

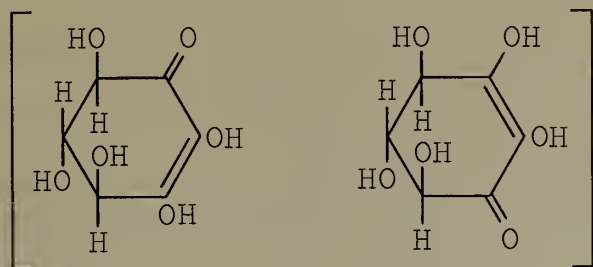
On oxidation, cyclohexanehexols (inositols) yield keto derivatives which, by successive enolization and elimination reactions, can be transformed into polyhydroxybenzenes [65]. In progressing from cyclohexane derivatives through cyclic olefinic intermediates to aromatic compounds, reactions and intermediate compounds are encountered which are eminently suited for the study of reaction mechanisms and basic concepts of theoretical organic chemistry.

In a recent publication [66], we reported the preparation and properties of D,L-xylo-trihydroxycyclohexenediolic acid (XXI) from the products of oxidation of myo-inositol with nitric acid. The structure of compound XXI was established, and certain unique and highly interesting reactions were found. The new compound reduces Tillmans reagent, reacts with iodine in neutral and acid solution, produces a blue color with a solution of ferric chloride, and exhibits other properties characteristic of the enediolic acid structure

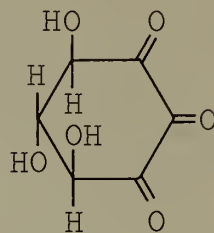
$$\begin{array}{c} \text{R}-\text{C}=\text{C}-\text{C}-\text{R}' \\ | \quad | \quad || \\ \text{HO} \quad \text{HO} \quad \text{O} \end{array}$$
 The compound is readily oxidized to xylo-

4,5,6-trihydroxy-1,2,3-cyclohexanetrione, an important new ketone. Compounds XXI and XXII are related to ascorbic acid and dehydroascorbic acid, respectively; like ascorbic acid

and dehydroascorbic acid, they are readily interconverted by oxidation and reduction.

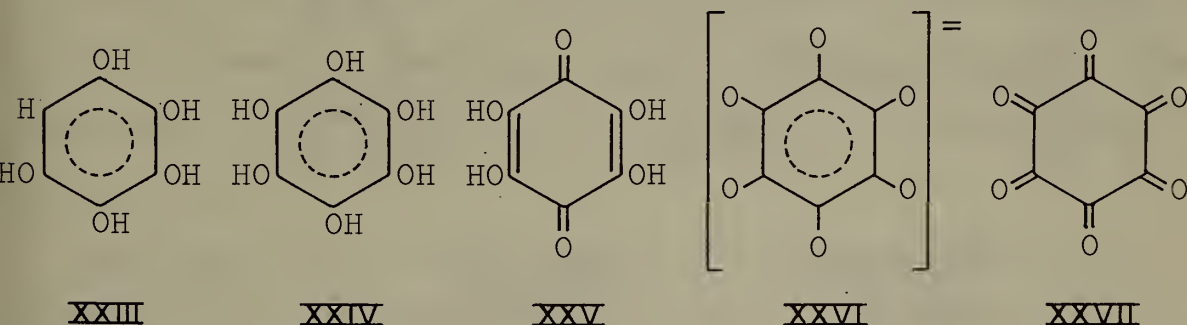


XXI



XXII

Acetylation of XXI and of XXII under basic conditions proceeds with simultaneous aromatization, resulting in the formation of pentaacetoxybenzene and hexaacetoxybenzene, respectively. On hydrolysis, the latter compounds yield pentahydroxybenzene (benzenepentol) (XXIII) and hexahydroxybenzene (benzenehexol) (XXIV), respectively. Oxidation of XXIV, by air in alkaline solution, yields tetrahydroxy-p-benzoquinone (XXV); further oxidation yields the anion of rhodizonic acid (XXVI) and cyclohexanehexone (XXVII) [65].



XXIII

XXIV

XXV

XXVI

XXVII

Synthesis of pentahydroxybenzene from myo-inositol provides a means for obtaining this highly interesting but little known polyphenol. Freshly prepared methanolic solutions of pentahydroxybenzene are slightly yellow; they

turn deep red on exposure to air. Ferric chloride solution acquires pink color that rapidly deepens to magenta. At room temperature, the compound reduces Benedict solution and, also, silver nitrate, the latter almost instantly. The compound may have useful medical applications.

Formation of pentaacetoxybenzene and hexaacetoxybenzene from XXI and XXII, respectively, was found to be in accordance with a mechanism previously advanced by Isbell [55] for the production of aromatic compounds from keto-inositols (see Figure 17). The mechanism involves successive enolization reactions, with cleavage of acyloxy groups beta to the enolic hydroxyl groups.

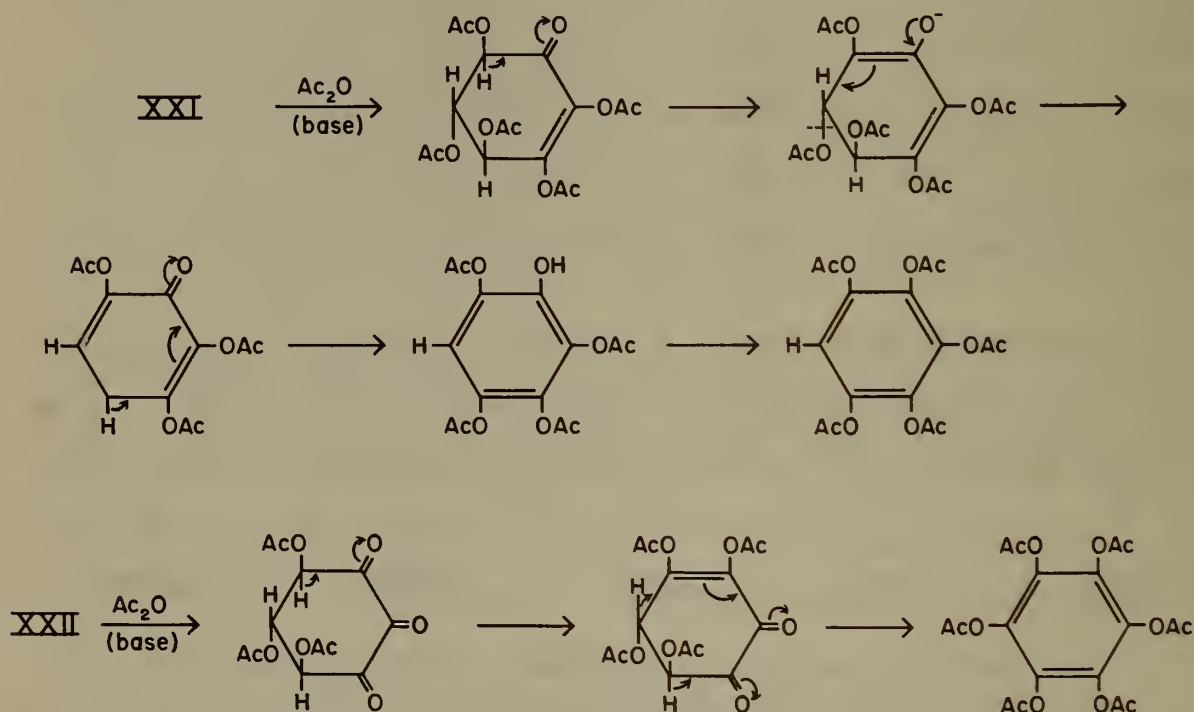


Figure 17. Mechanisms for the formation of pentaacetoxybenzene and hexaacetoxybenzene.

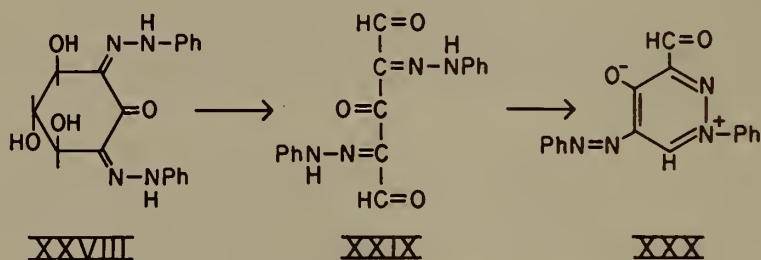
B. Phenylhydrazono-phenylazo Compounds

The structure of phenylhydrazono compounds in general is a subject of high current interest because, by use of spectrographic and resonance methods, some of the conventional structures have been shown to be incorrect. Thus, Mester [67] and others [68] have found that crystalline phenylosazones of sugars normally have a cyclic, chelate structure, first suggested by the Fiesers [69], instead of the conventional structure. Furthermore, it has been shown that certain phenylhydrazono compounds undergo complex tautomeric changes [70].

We have found that xylo-4,5,6-trihydroxy-1,2,3-cyclohexanetrione (XXII) (described in the preceding section of this report) forms a crystalline bis(phenylhydrazone) having some unusual properties. The bis(phenylhydrazone) obtained under mild conditions gives a yellow solution in toluene which turns red on standing. Evaporation of the toluene solution gives a dark-red, crystalline product. The change in color suggests a change from a phenylhydrazono to a phenylazo structure.

From the method of synthesis, the bis(phenylhydrazono) compound could be either a 1-oxo-2,3-bis(phenylhydrazone) or a 2-oxo-1,3-bis(phenylhydrazone) (XXVIII). Either compound should be capable of existing in tautomeric modifications. Hence, it was of interest to investigate the structures of XXVIII and related compounds in considerable detail.

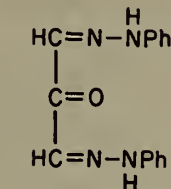
To determine the positions of the phenylhydrazono groups of XXVIII, we oxidized the red bis(phenylhydrazone) with sodium metaperiodate and separated the reaction product. It would be expected that the 2-oxo-1,3-bis(phenylhydrazone) would yield, by oxidation, the dialdehyde, XXIX. The initial oxidation product was found to rearrange, with cleavage of water, to a pyridazine derivative, XXX; this compound was obtained in good yield.



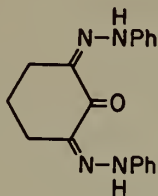
Formation of XXX established that the bis(phenylhydrazone) of xylo-4,5,6-trihydroxy-1,2,3-cyclohexanetrione is, indeed, 2-oxo-1,3-bis(phenylhydrazono)-xylo-4,5,6-trihydroxycyclohexane (XXVIII). The work also provided a route to new pyridazine derivatives having unusual structural features. The reactions and properties of these compounds are being studied.

After establishing the positions of the substituent groups in our 1,3-bis(phenylhydrazone), we turned our attention to the structures of the two forms of the substance. To ascertain whether other 2-oxo-1,3-bis(phenylhydrazones) likewise exist in tautomeric forms, we prepared and studied the following previously known compounds: 2-oxo-1,3-bis(phenyl-

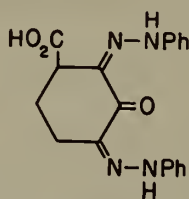
hydrazono)propane (XXXI); 2-oxo-1,3-bis(phenylhydrazono)cyclohexane (XXXII); 3-oxo-2,4-bis(phenylhydrazono)cyclohexane carboxylic acid (XXXIII); 2-oxo-1,3-bis(phenylhydrazono)cyclopentane (XXXIV); and 2-oxo-1,3-bis(phenylhydrazono)indan (XXXV).



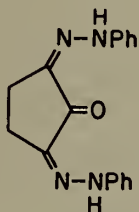
XXXI



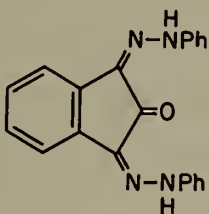
XXXII



XXXIII



XXXIV



XXXV

In each instance the product obtained by reaction of a trione with phenylhydrazine under mild conditions crystallized as a crude, yellow-orange mixture which turned red when heated in toluene (or other anhydrous solvent), and formed dark-red crystals on removal of the solvent.

The initial product from the reaction of 1,2,3-cyclohexanetrione and phenylhydrazine under mild conditions crystallized from aqueous ethanol as a yellow monohydrate. A freshly prepared, ice-cold, aqueous ethanol solution of the hydrate shows an absorption band at about 400 mμ which shifts rapidly to a longer wavelength (about 480 mμ). After equilibration and evaporation of the solvent, the red, anhydrous form of the compound is obtained. The IR, UV, and NMR spectra

of the hydrate indicate that it has a conventional phenylhydrazono structure. However, the spectra of the red modification of the same compound and the red forms of the other 2-oxo-1,3-bis(phenylhydrazones) listed above show that they are not true oxo-phenylhydrazones; they do not show infrared absorption in the regions characteristic of either the free carbonyl group or the imino hydrogen atom. All of the compounds give three characteristic absorption bands with λ_{max} 243 to 259, 283 to 290 and 468 to 488 μ , respectively. The similarity of the spectra support the conclusion that all of the red compounds have the same type of structure. The spectra are in accordance with the enolic, hybrid, phenylhydrazono-phenylazo structure depicted in Figure 18 for 2-oxo-1,3-bis(phenylhydrazono)cyclohexane (XXXII). The structure

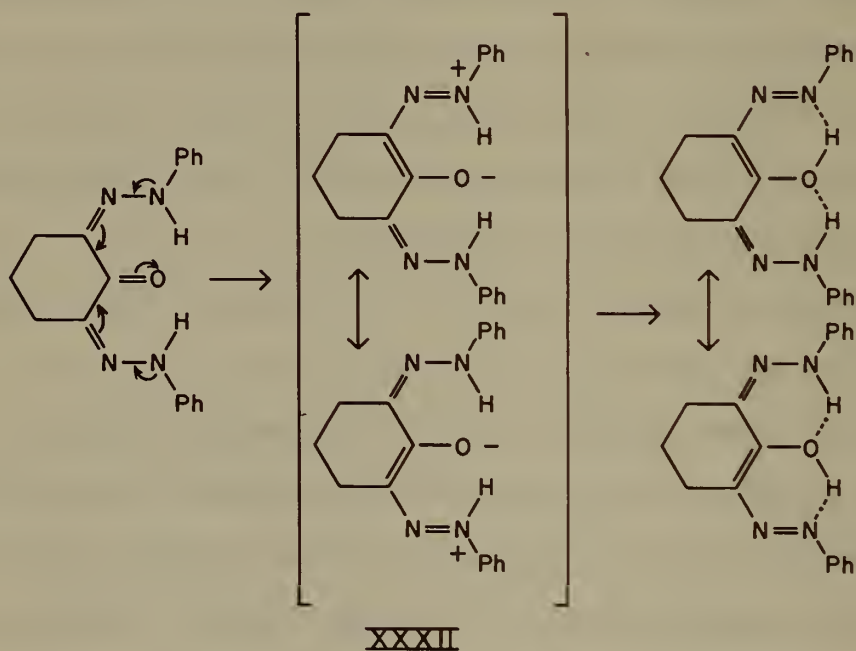


Figure 18. Proposed structure for a typical 2-oxo-1,3-bis(phenylhydrazono) compound.

of XXXII was further confirmed by its NMR spectrum in deuterated chloroform (see Figure 19). The observed low-field absorption, at about 14 ppm, is indicative of intramolecular hydrogen-bonding (presumably between the imino hydrogen atom and the carbonyl group).

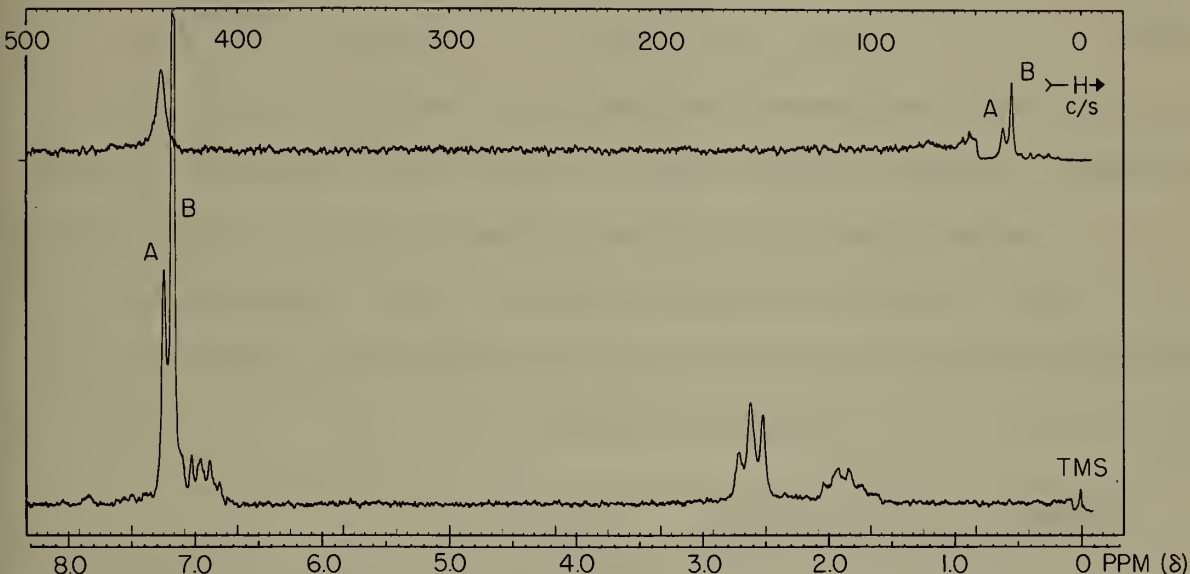
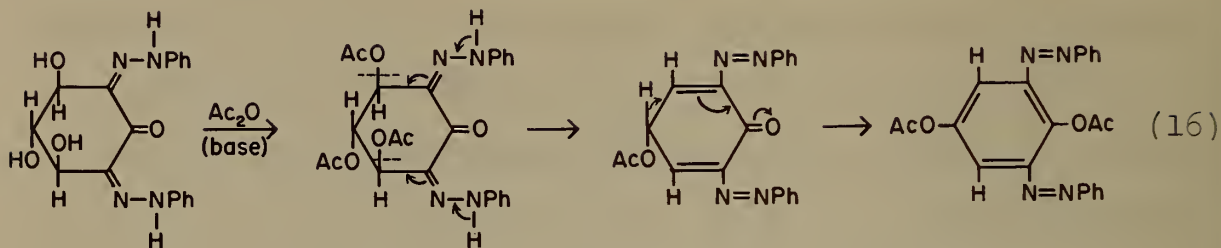


Figure 19. NMR spectrum of 2-oxo-1,3-bis(phenylhydrazono)cyclohexane (hybrid structure).

Confirmation of the enolic hybrid, phenylhydrazono-phenylazo structure for the red forms of compounds XXVIII, and XXXI - XXXV, inclusive, has been obtained from a comparison of the spectra of the compounds with those of model compounds having similar structural features. This work is now being prepared for publication.

The trihydroxycyclohexanetrione derivative XXVIII gives rise to many unusual reactions. By acetylation with a basic catalyst, it give 1,4-diacetox-2,6-bis(phenylazo)benzene

(XXXVI) in almost quantitative yield (see equation 16).



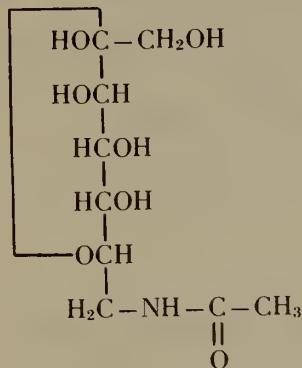
XXVIII

XXXVI

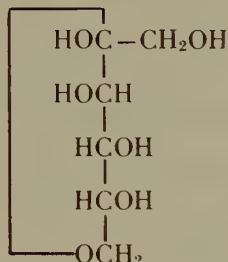
Compound XXXVI presumably arises from removal of the imino hydrogen of XXVIII by the action of the base catalyst followed by the changes depicted. The mechanism is based on the concept of successive, electron displacements [55] advanced by Isbell and used subsequently for the rationalization of many reactions in the carbohydrate field.

C. Acetamido-deoxyketoses

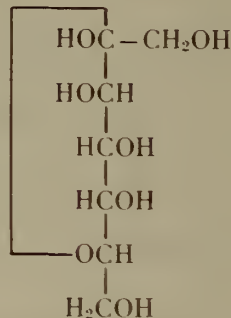
By oxidation of 1-acetamido-1-deoxy-D-glycero-D-galacto-heptitol with Acetobacter suboxydans, a new ketose (XXXVII), closely related to D-fructose (XXXVIII) and perseulose (XXXIX), was obtained [71]. The new sugar, 7-acetamido-7-



XXXVII



XXXVIII



XXXIX

deoxy-L-galacto-heptulose, was shown to be a pyranose by comparison of its optical rotation with that of β -D-fructopyranose.

Some of the properties of the new 7-acetamido-7-deoxy-L-galacto-heptulopyranose were determined for characterization. It melts at 139 to 140°C. With 1-butanol:pyridine:water (3:2:1) as an ascending, developing solvent, the mobility of the compound on Whatman No. 1 paper is 0.88 that of fructose. Color formation with staining reagents sensitive to ketoses is as follows: phloroglucinol, violet; urea phosphate, yellow (bright yellow fluorescence); resorcinol, tan (violet fluorescence); orcinol, purple (red fluorescence). The compound reacts with Somogyi copper-reducing reagent at approximately the same rate as does fructose.

The following equations represent the optical rotation of 7-acetamido-7-deoxy- α -L-galacto-heptulopyranose in water at 20°C and 3.5°C, respectively:

$$[\alpha]_D^{20} = -15.5e^{-.0396t} - 97.1,$$

$$[\alpha]_D^{3.5} = -11.9e^{-.0101t} - 105.8,$$

where t is the time (in minutes) after dissolution of the sample.

In conjunction with the synthesis of XXXVII, methods for the preparation of 1-amino-1-deoxyalditols were examined. Catalytic reduction of glycosylamines proved to be unsatisfactory, because of the instability of the glycosylamines in neutral or slightly acid solution [72]. Reduction of sugar oximes [73] was satisfactory, and this method is recommended

for small-scale preparations of 1-amino-1-deoxyalditols from readily available aldoses. Reduction of 1-nitro-1-deoxyalditols, prepared from the lower sugars by condensation with nitromethane [74], proved to be the most satisfactory method; reduction takes place readily, and gives the amines in high yield.

5. SYNTHESIS OF SELECTED COMPOUNDS

(R. S. Tipson and A. Cohen)

The purpose of this project is to devise chemical procedures for the synthesis of new organic compounds; to study mechanisms and kinetics of reactions involved in the preparation of these materials and of required intermediate compounds; and to determine the physical and chemical properties of the compounds, for use as reference materials. The work not only affords such materials for physical, chemical, and biological research, but serves in developing fundamental principles involved in organic reactions.

In one phase of this project, N,N-bis(2-chloroethyl)- and N,N-bis(2-iodoethyl)aniline and their hydrochlorides were prepared. Methods were devised for synthesis, in high yield, of the dibenzenesulfonate, dimethanesulfonate, and di-p-toluenesulfonate of 2,2'-(phenylimino)diethanol. Each compound was converted into the corresponding p-aldehyde. The interrelationships and structures of the compounds were proved, and their properties were determined. An article describing this work was published [75]. In an informal arrangement with the National Cancer Institute, each compound synthesized was submitted for evaluation of possible carcinostatic activity. The above-mentioned dimethanesulfonate was found to "have substantial activity in at least one test system."

We discovered [75] that, in the reaction of methane-

sulfonates with sodium iodide to give the corresponding iodo derivatives, the salt that is precipitated is not $\text{CH}_3\text{SO}_3\text{Na}$, but the double salt $4(\text{CH}_3\text{SO}_3\text{Na}) \cdot \text{NaI}$; this salt had been described by Collmann in 1868 [76]. Our discovery has since been confirmed [77]. The significance of the finding is that, in the past, various workers have used the weight of the salt formed as a measure of the extent of the reaction, and they had assumed that the salt was $\text{CH}_3\text{SO}_3\text{Na}$. Our work shows that an apparent yield of 132% represents an actual yield of 100% of $\text{CH}_3\text{SO}_3\text{Na}$, and that other apparent yields must be adjusted in the same ratio to provide the actual yield of $\text{CH}_3\text{SO}_3\text{Na}$.

Another phase of this project is concerned with derivatives of alditols as reference materials. Typical sulfonic esters of alditols have been prepared, and their physical and chemical properties have been determined. In particular, work has been done on the mechanisms and kinetics of nucleophilic substitution reactions and of elimination reactions in aprotic, dipolar solvents.

The initial work has been performed with sulfonic esters of D-mannitol, and a variety of derivatives of this alditol have been crystallized for the first time, including 3,4-di-O-(methylsulfonyl)-D-mannitol (XL) and 3,4-di-O-p-tolylsulfonyl-D-mannitol (XLI), and the 1,2,5,6-tetraacetates of esters XL and XLI. After purification of these products, their physical properties were determined; these properties included

melting point, specific optical rotation, and infrared absorption spectrum.

The rates of hydrolysis, by 80% acetic acid, of the isopropylidene groups from 1,2:5,6-di-O-isopropylidene-3,4-di-O-(methylsulfonyl)-D-mannitol (XLII) and 1,2:5,6-di-O-isopropylidene-3,4-di-O-p-tolylsulfonyl-D-mannitol (XLIII) were determined by methods involving optical rotatory power, and optimal preparatory methods for XL and XLI were developed. A simple method was devised for elimination of the sulfonyloxy groups in XLII and XLIII, thereby converting them into the corresponding acetal of trans-3-hexene-D-threo-1,2,5,6-tetrol, which was hydrolyzed by 80% acetic acid to the free, unsaturated tetrol, a compound of considerable interest. This tetrol and its di-O-isopropylidene acetal have been characterized for use as reference materials. The work will be extended to D-glucitol and other alditols.

Earlier work of the Section on acetamido derivatives has been extended. In collaboration with Professor Venancio Deulofeu, Buenos Aires, Argentina, we have examined the infrared spectra of some 40 l-acetamido derivatives of alditols and related compounds. Correlations have been made between absorption bands in the spectra and the structures of the various compounds. These results will be valuable for identification and in checking for purity of these compounds.

6. AIR POLLUTION STUDIES

(R. S. Tipson, A. Cohen, A. J. Fatiadi,
J. H. Gould, M. N. Inscoe, and H. S. Isbell)

This work is supported, in part, by the Division of Air Pollution, Public Health Service, U.S. Department of Health, Education, and Welfare.

A. Introduction

Pyrolysis or incomplete combustion of carbon-containing materials at high temperatures gives rise to polycyclic, aromatic compounds. Consequently, many compounds of this nature have been detected as pollutants in the air of large cities, particularly where dispersal is retarded.

Such air pollutants include many polycyclic, aromatic hydrocarbons and certain of their oxidation products. Some of these hydrocarbons are carcinogenic to animals and, possibly, to man. Eleven carcinogenic hydrocarbons have thus far been detected in automobile exhaust fumes; five are also present in practically all airborne particles (such as dust and soot) [78].

From particulate matter, obtained from polluted air, quinones of such hydrocarbons as pyrene, benzo[a]pyrene, and dibenzo[def,mno]chrysene, as well as other oxidized organic compounds (largely unidentified as yet), have been isolated and identified [79]. This suggests that, after release of reactive hydrocarbons into the atmosphere, some oxidation may normally occur in the air. Indeed, over thirty years ago, it was discovered [80] that, on exposure to air containing ozone,

the carcinogenic benzo[a]pyrene is transformed into less carcinogenic or noncarcinogenic material. Such oxidation is to be expected, as polluted air contains a number of oxidants: the nitrogen oxides, oxygen, and ozone of unpolluted air, together with such compounds as sulfur dioxide, produced by combustion.

The oxidation of polycyclic, aromatic hydrocarbons is also of interest because, in evolving ways for reducing air pollution, attention is currently being given to the development of chemical devices for oxidizing atmospheric pollutants produced by incomplete combustion and pyrolysis in automobile and Diesel exhaust fumes.

In considering the fate of these hydrocarbons and methods for minimizing their effects as air pollutants, it thus became of interest to assemble all of the available information on (1) the oxidants effective in the oxidation of polycyclic, aromatic hydrocarbons, (2) the relative reactivity of the hydrocarbons, (3) the conditions under which oxidation proceeds, (4) the chemical mechanisms involved when such oxidations occur, and (5) the products formed.

Therefore, a critical review entitled "Oxidation of Polycyclic, Aromatic Hydrocarbons" was written; this is to be published as NBS Monograph 87 [81]. As part of our study on the problem of air pollution, an attempt will be made to maintain a current bibliography of pertinent scientific literature on

this subject, with the object of serving our group and others.

B. Interaction of Aromatic Hydrocarbons with Oxygen under Pressure

To gain an understanding of the oxidation of polycyclic, aromatic hydrocarbons, information regarding their behavior with oxygen under pressure is a useful first step. Consequently, we have observed the spectral changes produced by the interaction of a number of aromatic hydrocarbons with oxygen at a number of pressures and temperatures, relative to a reference spectrum of the sample treated with nitrogen.

1. Apparatus

In order to be able to investigate the effect of temperature and pressure on the reaction of oxygen with the aromatic hydrocarbons, we devised and constructed a pressurizing system in an absorption-cell thermostat that we had previously constructed for use with the Cary Model-14 Spectrophotometer. In its working range of +60 to -100°C, this sample-cell thermostat maintains the temperature of the sample within a few degrees C. Simultaneously, the solution in the absorption cell is subjected to a fixed, moderate pressure of the chosen gas (to date, oxygen; but such gases as nitric oxide and sulfur dioxide could be used). The upper limit of the gas pressure used has been thirty pounds per square inch (gauge); a limit set by the strength of the ordinary absorption cells employed.

The apparatus is shown in Figures 20 and 21. It employs

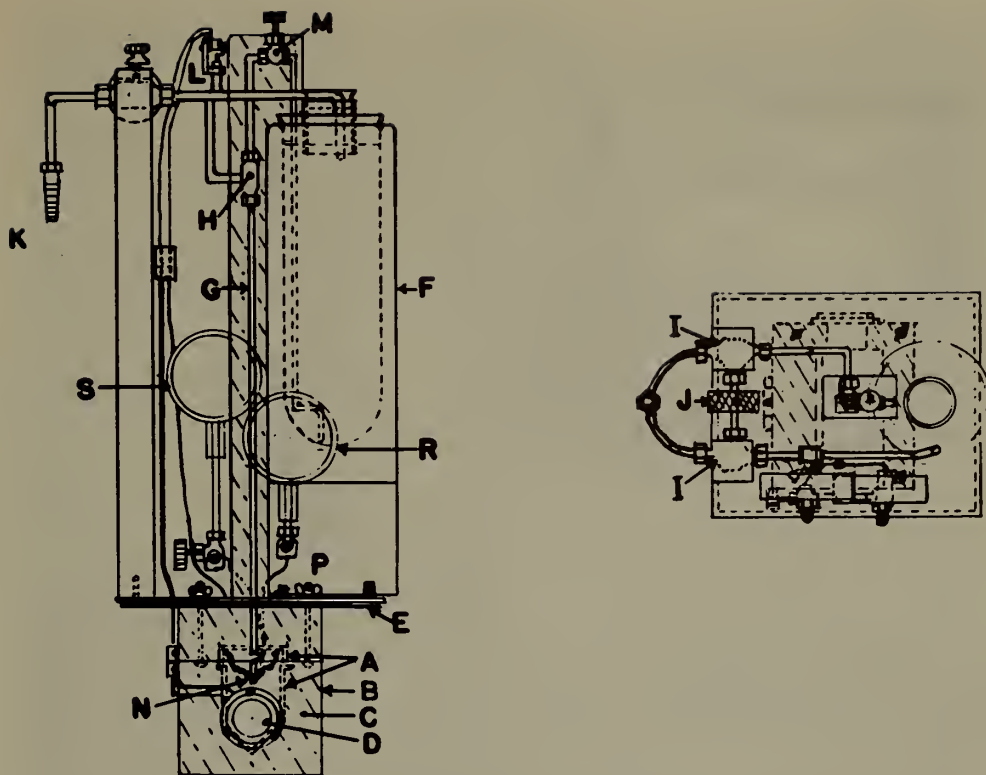


Figure 20. Diagram of cell for spectrophotometric study of the interaction of hydrocarbons with gases.

a V-bottomed, metal cell-container (A) which aligns the absorption cell in the sample-beam of the spectrophotometer. Container A is held inside a metal box (B) by means of rigid, Styrofoam insulation (C). Both A and B are fitted with quartz windows (D), which are insulated from the walls by cork gaskets. Both sets of windows are in contact with electric heaters which prevent the formation of fog or frost at the lower temperatures employed. Moreover, these heaters are the primary source of heat when the system is operated above room temperature.

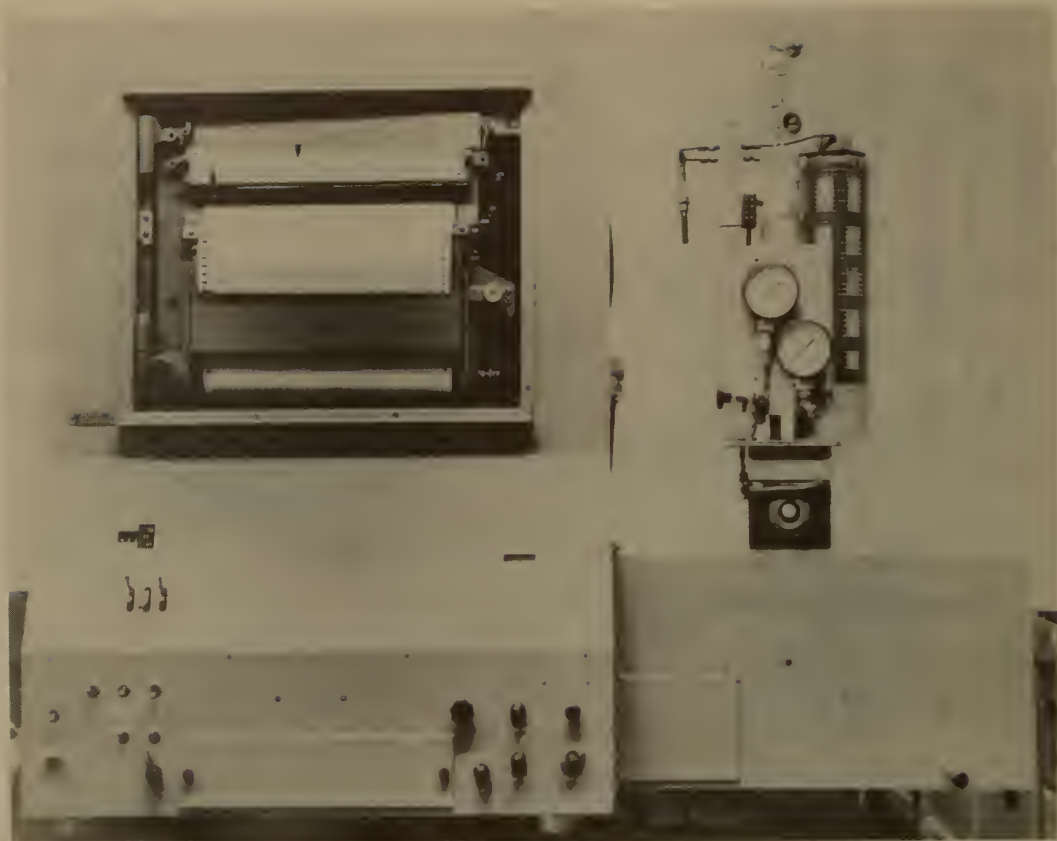


Figure 21. Photograph of apparatus for the spectro-photometric study of the interaction of hydrocarbons with gases.

The sample compartment is attached below a replica (E) of the sample-compartment cover by four studs and is secured by four wingnuts. To the top of E is fastened a one-liter Dewar flask (F); this contains the liquid nitrogen used as the refrigerant, and is stoppered. The fluid for transfer of heat is dry air at low pressure or tank nitrogen, admitted by the hose connection (K). This gas stream is split in two, and passes through two identical needle-valves (I) which are coupled, back to back, by a knob (J). This arrangement ensures

that (1) the valves will operate in an inverse manner, and (2) the total flow through the apparatus will remain about the same, regardless of the distribution of the heat-transfer gas. It also provides a crude means of setting the response of the system to the action of the temperature-control system.

The first stream of gas is conducted directly to the sample-compartment via the mixing chamber (H) and tube (G), both of which are insulated with Styrofoam. After the gas has bathed the absorption cell, it is vented to the atmosphere through a tube (not shown).

The second stream of gas is admitted into the top of the Dewar flask (F) past an electrically operated valve (L) which is either open to the atmosphere or closed, depending on the degree of cooling to be applied to the sample in order to maintain the low temperature selected. When cooling is required, valve L closes and the liquid nitrogen is forced from F, through the control valve (M), and into the mixing chamber (H), where it evaporates, thereby cooling the primary stream and, subsequently, the absorption cell.

The open or closed condition of valve L is determined by the temperature-control system; this consists of two copper-constantan thermocouples (one for the sample and one as a reference), a potentiometer, and an amplifier whose output operates valve L. The thermocouple for the sample is pressed against the top of the absorption cell by means of a spring. The reference junction is kept in an ice bath. The opposed

output from the thermocouples is applied, through a reversing switch, to the input of the potentiometer. This potentiometer, which is the primary temperature-measuring device, also acts as the balance unit for the control system, because the input to the amplifier is obtained from the galvanometer of the potentiometer. The amplifier is a Brown Recorder amplifier, so modified as to provide a single-ended output of reversible phase. The AC output of the amplifier is rectified, and then applied to the control valve L, so as to inject or withhold refrigerant as determined by the direction of the current through the galvanometer.

After the proper setting of the phasing switches has been achieved, the temperature of the absorption cell can be set, and maintained, merely by turning the potentiometer dial to the millivolt reading required for production of the desired temperature.

By means of a second system, the solution under study is treated with a gas under pressure. In operation, the pressure-regulated gas is admitted, from a tank, into the absorption cell, by means of a spring-loaded, injector assembly (N). The gas is conveyed from the tubing connections (P) by capillary tubing. The upstream and downstream pressures are recorded by gauges (R) and (S), respectively.

The seal at the neck of the absorption cell is provided by a stopper that had been cast, on a paraffin block, from a silicone rubber vulcanizing at room temperature.

2. Results

Figure 22 shows the changes observed in the spectrum of a solution of benzene in 2-methylheptane on treatment with oxygen, relative to the spectrum of the same solution on treatment with nitrogen. The fine structure present is attributed to singlet-triplet transitions [82], and the general absorption underlying it is the result of charge transfer between the benzene (donor) and the oxygen (acceptor).

To date, we have made observations on the spectral changes produced by the interaction of oxygen with solutions of naphthalene, anthracene, naphthacene, phenanthrene, benz[a]anthracene, and pyrene in inert solvents, relative to the spectra of the same solutions treated with nitrogen. The magnitude of the changes produced by oxygen is a function of the oxygen pressure and the concentration of the aromatic hydrocarbon.

Although the spectral changes observed for the polycyclic hydrocarbons mentioned are not so spectacular as for benzene, either in magnitude or complexity, they are readily observed. With the exception of naphthacene and benz[a]anthracene, the oxygen-induced absorption produced in the spectrum of each of these hydrocarbons is (a) completely removed on displacing the oxygen by treatment with nitrogen, and (B), in intensity, directly proportional to the absolute pressure of oxygen applied.

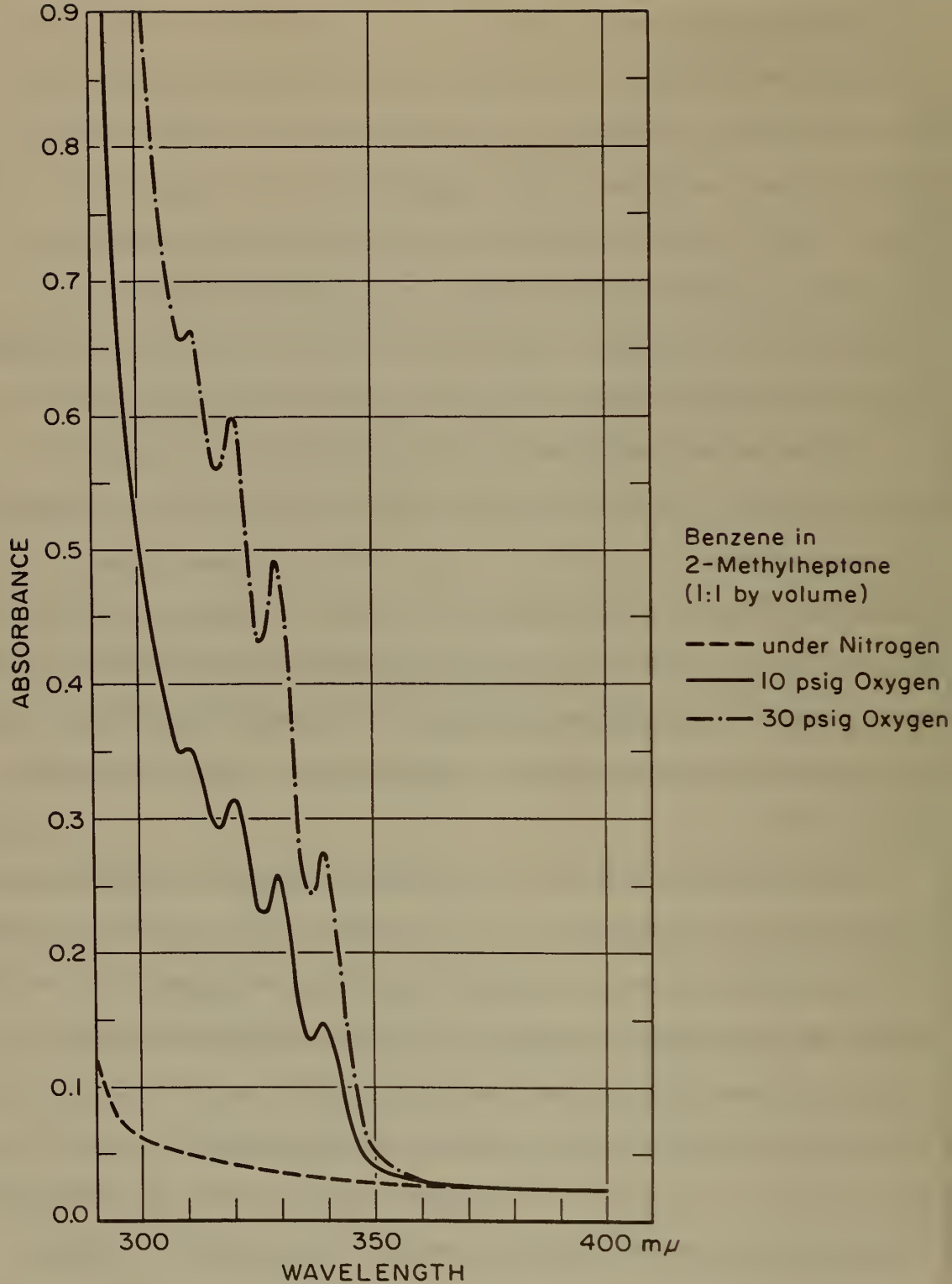


Figure 22. Spectral changes induced by treatment of benzene with oxygen.

However, for naphthacene, the absorption does not appear to be proportional either to the pressure or the square of the pressure (over the whole range of available pressures). In addition, naphthacene gives a large response to oxygen treatment relative to its concentration, as compared with the behavior of the other hydrocarbons studied.

For naphthacene and benz[a]anthracene, we observed non-reversibility on treating the oxygenated solutions with nitrogen. (Such nonreversibility was not observed for the spectra of the other hydrocarbons studied.) It indicates the formation of a permanent chemical compound of the hydrocarbon with oxygen under pressure. For naphthacene, formation of this compound was promoted by irradiation with light from a tungsten source or from the near-infrared source of the Cary Model-14 spectrophotometer. Whether such promotion can be effected for the other hydrocarbons has not yet been studied.

C. Oxidation of Polycyclic, Aromatic Hydrocarbons on Particulate Matter

Observations made during the application of thin-layer chromatography to characterization and determination of purity of various polycyclic, aromatic hydrocarbons led to our discovering that alterations which take place in the appearance of the spots are often the result of photochemical reactions on the thin-layer chromatograms [83].

Observations were made on spots of 15 representative hydrocarbons following exposure to ultraviolet light. The

adsorbents used were silica gel G, aluminum oxide G, cellulose powder, and acetylated cellulose (21%). Four of the hydrocarbons (phenanthrene, chrysene, triphenylene, and picene) show no changes after such exposure, other than a slight fading of fluorescence when allowed to stand in room light for several days. On silica gel G and aluminum oxide G, pronounced changes occur, both in the appearance and in the fluorescence of the 11 other hydrocarbons. Spots of the hydrocarbons having an anthracenic structure (anthracene, naphthacene, benz[a]anthracene, dibenz[a,c]anthracene, and dibenz[a,h]anthracene) turn yellow or a yellow-tan; spots of condensed hydrocarbons (pyrene, benzo[a]pyrene, benzo[e]pyrene, perylene, benzo[ghi]perylene, and coronene) turn darker tan or brown. At the same time, the appearance of the spots under ultraviolet illumination also changes. The fluorescent color characteristic of each hydrocarbon (usually a shade of blue or green) becomes dull and is gradually lost, while the spots take on an orange or red coloration, usually changing to such a deep red that they appear completely dark. A halo (blue, green, orange, or red, depending upon the hydrocarbon involved) is often seen at the edge of the dark spot. On powdered cellulose or acetylated cellulose, the behavior of these 11 hydrocarbons is somewhat similar, although the changes in appearance and fluorescence are much less extensive and develop much more slowly.

After the initial exposure to ultraviolet light, these changes take place even when the plates are kept in the dark. Similar, but slower, changes also occur on plates kept in ordinary room-light, without exposure to other ultraviolet illumination. The changes are accelerated by continuous irradiation, either by long-wavelength ultraviolet light or by light of 253.7 mμ.

The nature of the developing solvent appears to have little effect on the colors observed. However, although it has sometimes been recommended that the chromatoplates be read while they are still wet [84,85] we have found that the presence of solvent often accelerates the changes in the spots. This effect is particularly noticeable with chlorinated solvents. For example, on a plate coated with silica gel G and developed with carbon tetrachloride, a pyrene spot, examined under ultraviolet light while still wet, turns dark and loses much of its fluorescence within 5 minutes. However, if the plate is allowed to dry for 10 to 15 minutes in the dark before being exposed to ultraviolet light, the spot is more stable and the blue-green fluorescence is still strong after several hours, although by this time some darkening of the spot can be observed in visible light.

In some instances, this darkening can be utilized in the visualization and identification of hydrocarbon spots. Benzene solutions of pyrene, benzo[a]pyrene, benzo[e]pyrene, and benzo[ghi]perylene were separately spotted on a silica gel G

plate (about 10 μ g of hydrocarbon per spot), developed with heptane, dried in the dark for 15 minutes, and exposed to continuous, long-wavelength ultraviolet illumination. The pyrene and benzo[a]pyrene spots turned brown within 5 minutes, and all four spots were easily visible in room light after 15 minutes of ultraviolet irradiation. In another experiment, the gradual appearance of a brown spot on a silica gel G chromatogram aided in demonstrating the presence of pyrene as an impurity in a sample of chrysene.

The reactions which give rise to these changes in the hydrocarbon spots are undoubtedly quite complex. Presumably, such photo-oxidations as have been reported for anthracene on alumina or silica gel [86] are involved. This is supported by our identification of 1,6-pyrenedione and 1,8-pyrenedione among the numerous products of the reaction of pyrene on silica gel G.

The work is being extended to oxidation, on a larger scale, on particulate matter in the presence of air and ultraviolet light. For separation, identification, purification, and quantitative determination of the products formed, column chromatography and gas—liquid chromatography are being used, in conjunction with ultraviolet, infrared, and nuclear magnetic resonance spectroscopy.

D. Determination of Losses Due to Adsorption, During
Filtration of Aqueous Solutions of Polycyclic, Aromatic
Hydrocarbons

In the course of some preliminary experiments on solubilization, undertaken by us two years ago, erratic results were obtained for the solubilities of pyrene and benzo[a]pyrene in aqueous solutions of polyvinylpyrrolidone (PVP) or deoxyribonucleic acid (DNA); these were traced to adsorption of the hydrocarbons by the filter media.

Although it has been pointed out that some adsorption of a solute may occur during filtrations [87], there is apparently no general awareness of the possible magnitude of such losses with dilute solutions of certain solutes. Loss by adsorption of polycyclic, aromatic hydrocarbons during filtration [88] has been mentioned briefly, and such adsorption has been shown [89] to be the cause of a major discrepancy in some recent attempts [90] to determine the solubilities of such hydrocarbons in aqueous solutions of DNA. However, there has been no detailed report on the extent of losses due to adsorption.

To evaluate the extent of this adsorption, studies were initially made on solutions of phenanthrene in distilled water, because phenanthrene is sufficiently soluble in water to permit changes in concentration to be observed spectrophotometrically by means of the strong absorption band at 251 mμ. The solutions used had been pre-filtered, usually through a membrane filter

(Polypore 27A; see Table 6, footnote c), and, on visual examination in ultraviolet light [91], showed no particles of undissolved phenanthrene. All spectrophotometric measurements were made on a Cary Model-14 spectrophotometer, using matched, fused quartz, absorption cells with the appropriate solvent or solution as a reference. To reduce errors arising from the background absorption caused by (a) ultraviolet-absorbing materials leached from the filters and (b) light-scattering by suspended particles, the base-line technique [92] was used for determinations of concentration [Note 1].

The results are shown in Table 6, from which it may be seen that, during filtration, phenanthrene is adsorbed from aqueous solutions by various filter media. Losses are particularly pronounced when membrane filters are used. The amount of hydrocarbon removed from solution by adsorption during filtration depends to some extent on the composition of the filter, but the pore size of the filter also has a very marked effect, indicating the influence of changes in surface area as well as in the rate of filtration. The pronounced increase in the amount of hydrocarbon removed from solution when two filters are used instead of one (see the data obtained with Polypore 27A) is also a result of both increased surface area and decreased rate of flow. The effect of changes in the rate of filtration alone may be seen particularly well in the data for Polypore AM-1 and for Polypore GM-6, where a decrease in the rate of filtration resulted in a sharp decrease in the

Table 6. Changes in concentration of phenanthrene in filtrate, during filtration of solutions of phenanthrene in distilled water.

Filtrate Fractions ^a											
	Pore Size	Orig. Conc.	First		Second		Third		Fourth		
			Vol. ml	Conc. % ^b	Vol. ml	Conc. % ^b	Vol. ml	Conc. % ^b	Vol. ml	Conc. % ^b	
Glass		Mx10									
coarse frit	40-60	6.5	15	84	15	96	60	99			
fine frit	4-5.5	6.9	15v	64	15v	84	60v	96			
Paper											
Whatman No. 1		5.3	15	56	15	74	60	87	60	93	
Whatman No. 2		4.5	15	24	15	50	60	74	60	79	
Membrane filter-holder ^c (sintered, stainless steel)		6.8	15	54	15	87	60	96			
Millipore ^d											
HA	0.45	7.3 ^e	140	0	50	2	50	7	80v ^f	42	
OH (solvent-resistant)	1.5	6.9	15	1	50	14	100	26	365	41	
Polypore ^c											
AM-1	5	6.1 ^e	50v ^g	41	50v ^h	31	50v ^g	64	540v	79	
AM-3	2	3.5	25	0	50	0	50	0	50v	17	
AM-5	0.65	6.4 ^e	50v	0	50v	2	50v	6	50v	14	
AM-7	0.3	7.8 ^e	75v	0	75v	0	75v	0	340v	0	
GM-6 (gravimetric)	0.45	5.9	50v	28	50v	53	50	32	50v	66	
27A (solvent-resistant)	0.45	7.5 ^e	15	2	50	70	50	91	50v		
27A (two membranes superimposed)	0.45	7.9 ^e	15	0	60	0	75	1	75	24	

^aBy gravity except where designated "v" (vacuum).

^bPer cent of the original concentration.

^cFrom Gelman Instrument Co. (1962); 2-inch diam.

^dFrom Millipore Filter Corp.; 47-mm diam.

^eA suspension, not necessarily saturated.

^fAfter filtration of three 50-ml portions.

^gFiltered rapidly.

^hFiltered more slowly, by lessening the vacuum.

concentration of phenanthrene in the filtrate.

Losses were also noted when aqueous solutions of naphthalene, pyrene, or anthracene were filtered, the relative losses being greater with the less-soluble hydrocarbons. Thus, when an aqueous solution of pyrene was filtered through a sintered-glass filter (fine frit), the concentration of pyrene in the first 50-ml portion of filtrate was 44% of the original concentration. Also, no anthracene could be detected spectrophotometrically in the final 20-ml portion of filtrate after filtration of 270 ml of an aqueous suspension of anthracene through Polypore AM-7.

When solutions of pyrene, benzo[a]pyrene, benz[a]anthracene, dibenz[a,h]anthracene, or similar hydrocarbons in aqueous solutions of PVP or DNA were filtered, there was a marked loss of hydrocarbon in the filtrate. No adsorption of hydrocarbon was observed from solutions in aqueous ethanol (20 or 50%) or in other organic solvents.

Such polymeric materials as PVP and DNA were also adsorbed from aqueous solution, but the filters were quickly saturated by these materials and, after the first 20 to 50 ml of filtrate had been collected, further changes in concentration of the filtrate were not usually observed. In contrast to this rapid saturation, and the saturation of membrane filters by protein [93], the filters are not readily saturated by the hydrocarbons. Occasionally, in addition to the errors introduced by this adsorption [Note 2] during filtration, particles of some hydro-

carbons (seen as bright, fluorescent specks when the filtrates are viewed in ultraviolet light) pass through paper or sintered-glass filters. However, the centrifugation procedure of Kofahl and Lucas [95] was found to give fairly reproducible solutions of hydrocarbons even in viscous solutions containing highly polymerized DNA. Two centrifugations, with Pyrex-wool plugs centrifuged down onto the residue, were sufficient to ensure removal of suspended hydrocarbon. Comparison of the spectra of the resulting solutions and of the original suspensions, using the base-line technique, showed the change in concentration of the dissolved hydrocarbons, due to centrifugation, to be less than 10%. By this method, considerably less variation in concentration is observed than when filtration is used to remove undissolved hydrocarbon. The loss may arise from adsorption on the centrifuge tubes and on the glass-wool plugs.

Note 1. It is especially important to use this method in solubilization experiments if the solvent is a solution of a polymeric material. The hydrocarbon often appears to bring about changes in the light-scattering and sedimentation properties of the polymer, with the result that the background absorption of the reference blank and the experimental solution are no longer exactly comparable. On applying the base-line technique to the spectral curves shown in Fig. 3 of reference [90] and estimating the

concentration from readings at 385, 395, and 405 mμ, we have found that the concentration of benzo[a]pyrene remains quite constant (approximately 1×10^{-6} M) during the centrifugation. The spectral changes observed appear to be the result of the more rapid sedimentation of the DNA in the hydrocarbon-containing solution than in the solution of DNA only (cf, [88]). We have observed similar effects with PVP solutions of aromatic hydrocarbons.

Note 2. Munck [94] used silicone-treated glassware to lessen the adsorption of steroids from aqueous solutions, but in the present work, similar treatment of fritted-glass filters with a silicone preparation (Desicote, Beckman Instrument Co.) did not produce any clear-cut effect on the amount of phenanthrene adsorbed during filtration. The reduced affinity of the treated surface for the hydrocarbon appears to be counteracted by the effect of the reduction in the rate of filtration brought about by the hydrophobic surface of the filter.

E. Preparation of Reference Compounds; Purification by Column Chromatography

For use in identification of materials by thin-layer chromatography and gas—liquid chromatography, samples of pure reference compounds are needed, so that their behavior can be established.

For each hydrocarbon whose oxidation is studied, a number of oxidation products that might possibly be formed under the particular conditions of oxidation are required. Thus, in connection with studies on the oxidation of pyrene, the following reference compounds were prepared: 1-, 3-, and 4-pyrenol, 1,6-, 1,8-, and 4,5-pyrenediol, and 1,3,6,8-pyrenetetrol.

In addition, methods have been developed for separation of the pyrenediones by column chromatography [96]. The 1,6-pyrenedione was separated from 1,8-pyrenedione on a column of silica gel, with glacial acetic acid as the eluant. The 1,6-pyrenedione was then finally purified on a column of activated alumina, with benzene as the eluant. The properties (including ultraviolet and infrared spectra) of purified 1,6- 1,8-, and 4,5-pyrenedione and of 1-oxo-6,7-phenalenedicarboxylic anhydride were determined.

7. REFERENCE MATERIALS (ORGANIC)

(H. S. Isbell and Staff)

A. Rare Organic Compounds

The development of numerous instrumental methods of analysis whereby substances are determined both qualitatively and quantitatively by comparison of an unknown with a standard has created a strong demand for a wide variety of reference materials. During the past year, our Section has fulfilled 21 requests from research workers elsewhere for small samples of rare compounds prepared in the course of our research program and not available elsewhere. The compounds have been of great value to these research workers, as shown by letters expressing appreciation for the service. During the coming year, we plan to improve and extend this service.

B. ^{14}C and ^3H -Labeled Carbohydrates

Under our labeled carbohydrate project, we have maintained a supply of the labeled carbohydrates and have furnished research workers in other institutions with 43 samples (5250 μc) during the current year.

C. Sugar Standards

Production and distribution of standard samples of dextrose and sucrose are being continued. During the year, 458 samples of the sugar standards were sold.

D. Metallo-organic Standards

Preparation and distribution of metallo-organic standards

is being continued. During the year, 1332 samples of metallo-organic standards were sold. Our supplies of ferric cyclohexanebutyrate (Standard Reference Material No. 1058) and of tris(2'-hydroxyacetophenono)chromium (III) (Standard Reference Material No. 1072) were exhausted. Inasmuch as the compounds used as the earlier standards are slightly hygroscopic, we reinvestigated the derivatives of 1-phenyl-1,3-butanedione for use as new standards. Measurements of the changes in weight of the materials used as the earlier standards and of the new 1-phenyl-1,3-butanedione chelates showed that the latter are less susceptible to change in weight on exposure to air under laboratory conditions. Hence, 1-phenyl-1,3-butanedione chelates were used for new standards for iron and chromium. Procedures for preparation of the new standards, and for preparing stable solutions of the compounds, are given in the sections that follow.

1. Tris(1-phenyl-1,3-butanediono)chromium(III) (Standard Reference Material No. 1078)

Chromium(III) chloride hexahydrate (293.1 g, 1.1 moles) is dissolved in 1200 ml of water in a 5-liter flask equipped with a stirrer and heated in an oil bath. The mixture is vigorously stirred while a solution of 487 g (3 moles) of 1-phenyl-1,3-butanedione in 2000 ml of ethanol is slowly added. The resulting solution is heated and stirred while a solution of 200 g of urea in 350 ml of water is added during

30 minutes. The mixture is heated and stirred overnight, after which the precipitate is collected and washed with hot water until the aqueous washings are colorless. Finally, the solid is washed with ethanol and air-dried; weight, 456 g (85%). About sixty g of unreacted diketone may be recovered from the filtrate.

The dry, crude product is dissolved in 2000 ml of chloroform and stirred while 1000 ml of ethanol is added. The mixture is stored overnight in a refrigerator. The crystalline product is separated by filtration, recrystallized from chloroform as before, dried, and powdered (100 mesh).

Anal. Calcd. for $C_{30}H_{27}CrO_6$: Cr, 9.71. Found: Cr, 9.58.

2. Tris(1-phenyl-1,3-butanediono)iron(III). (Standard Reference Material No. 1079).

1-Phenyl-1,3-butanedione (502.7 g, 3 moles) is dissolved in 1500 ml of absolute ethanol in a 3-liter flask, and vigorously stirred while 1200 ml of concentrated ammonium hydroxide is slowly added. The resulting solution is added, with stirring, to a hot solution of ferric chloride hexahydrate (270.2 g, 1 mole) in 400 ml of water. The dark-red compound begins to separate immediately. The mixture is stored overnight in a refrigerator, and the resulting crystals are separated by suction filtration, and washed, first with hot water, and then with ethanol. The air-dried, crude compound weighs about 500 g (92%).

The compound is recrystallized from 1500 ml of hot chloroform by addition of 900 ml of warm ethanol. Crystallization begins after about 10 minutes, and is allowed to continue overnight. The crystals are collected on a filter, air-dried, and recrystallized as before from chloroform. The yield of the pure product is about 444 g (82%).

Anal. Calcd. for $C_{30}H_{27}FeO_6$: Fe, 10.35. Found: Fe, 10.26.

The new standard reference materials are crystalline, and are easily prepared in high yield. They are relatively stable in the presence of moisture. After exposure of a 0.5-g quantity of each compound to 75% humidity for 120 hours, the iron derivative gained 0.3 mg and the chromium derivative gained 0.2 mg. Both samples readily lost this moisture on being heated for 1 hour at 110°C .

The solubilities of these new standards in lubricating oils are increased by the use of 6-methyl-2,4-heptanedione and 2-ethylhexanoic acid, the reagents used previously for Standard Reference Materials 1058 and 1072. The solutions prepared with these reagents are stable, and compatible with the other NBS metallo-organic standards.

8. PUBLICATIONS, REPORTS, AND LECTURES

A. Publications

Tritium-labeled Compounds. X. Isotope Effects in the Oxidation of Aldoses-l-t with Bromine.

H. S. Isbell and L. T. Sniegoski, J. Res. NBS 68A, (Phys. and Chem.) No. 2, 145 (1964).

Cyclic Polyhydroxy Ketones. II. xylo-Trihydroxycyclohexenediolic Acid and Keto-inositols.

A. J. Fatiadi and H. S. Isbell, J. Res. NBS 68A, (Phys. and Chem.) No. 3, 287 (1964).

Tritium-labeled Compounds. XI. Mechanism for the Oxidation of Aldehydes and Aldoses-l-t with Sodium Chlorite.

H. S. Isbell and L. T. Sniegoski, J. Res. NBS 68A, (Phys. and Chem.) No. 3, 301 (1964).

Synthesis of Higher Ketoses by Aldol Reactions. II. Unsubstituted Heptuloses.

R. Schaffer, J. Org. Chem. 29, 1471 (1964).

The Isomerization of D-manno-3-Heptulose by Alkali.

R. Schaffer, J. Org. Chem. 29, 1473 (1964).

Report on Sugars and Sugar Products - October 1963.

E. J. McDonald, J.A.O.A.C. 47, 69 (1964).

Photochemical Changes in Thin-layer Chromatograms of Polycyclic, Aromatic Hydrocarbons.

M. N. Inscoe, Anal. Chem. 36, 2505 (1964).

Methods in Carbohydrate Chemistry, Vol. 5, Academic Press, (1965). (64) Determination of Reducing End-groups by

Reaction with C¹⁴-Labeled Cyanide.

H. S. Isbell.

Synthesis and Ring Structure of 7-Acetamido-7-deoxy-L-galacto-heptulose.

E. J. McDonald, J. Res. NBS 69A, (Phys. and Chem.) No. 3, 291 (1965).

Deuterium Isotope Effects in the Mutarotations of α -D-Glucose and β -D-Fructose.

Ph.D. Thesis by Clarence W. R. Wade, submitted to Georgetown University, May 14, 1965.

B. Manuscripts Accepted for Publication

Oxidation of Polycyclic, Aromatic Hydrocarbons.

R. S. Tipson. Accepted for publication as NBS Monograph 87.

Separation of Pyrenediones by Column Chromatography.

A. J. Fatiadi. Accepted for publication in Journal of Chromatography.

Synthesis of D-glucose-3-¹⁴C and Related Compounds.

H. L. Frush, L. T. Snięgoński, N. B. Holt, and H. S. Isbell.
Accepted for publication in Journal of Research, NBS.

C. NBS Reports

(Quarterly Reports on Air Pollution Program prepared jointly with certain Sections of the Physical Chemistry Division)

NBS 8360 - Quarterly Report AIR POLLUTION PROGRAM -

1/1/64-3/31/64. P. J. Ausloos, H. S. Isbell, R. Klein, R. Stair, R. S. Tipson.

NBS 8363 - Special Report AIR POLLUTION PROGRAM - 4/27/64 -

R. S. Tipson.

NBS 8512 - Quarterly Report AIR POLLUTION PROGRAM -

4/1/64-6/30/64. H. S. Isbell, R. Klein, R. E. Rebbert, C. A. Samson, R. Stair, R. S. Tipson.

NBS 8582 - Quarterly Report AIR POLLUTION PROGRAM -

7/1/64-9/30/64. H. S. Isbell, R. Klein, R. E. Rebbert, R. Stair, R. S. Tipson.

NBS 8634 - Quarterly Report AIR POLLUTION PROGRAM -

10/1/64-12/31/64. H. S. Isbell, R. Klein, R. E. Rebbert, R. Stair, R. S. Tipson.

NBS 8683 - Quarterly Report AIR POLLUTION PROGRAM -

1/1/65-3/31/65. H. S. Isbell, R. Klein, R. E. Rebbert, R. Stair, R. S. Tipson.

NBS 8930 - Quarterly Report AIR POLLUTION PROGRAM -

4/1/65-6/30/65. R. Klein, R. E. Rebbert, R. Stair, R. S. Tipson.

D. Lectures

- 1/6/64 - Nomenclature of Sugar Conformers. Chemistry Seminar - Georgetown University, Washington, D. C. - R. S. Tipson
- 1/13/64 - Higher Sugar Synthesis by Aldol Reactions. Chemistry Seminar - Georgetown University, Washington, D. C. - R. Schaffer
- 1/20/64 - Isotope Effects in Chemical and Biological Reactions. Chemistry Seminar - Georgetown University, Washington, D. C. - H. S. Isbell
- 5/1/64 - Phenylhydrazono-phenylazo Structures for 2-Oxo-1,3-Bis(phenylhydrazono) Derivatives. Bi-sectional ACS regional meeting, University of Maryland - A. J. Fatiadi and H. S. Isbell
- 5/28/64 - Branched-chain Monosaccharides. Fifth Annual Medicinal Chemistry Symposium, University of New York at Buffalo - R. Schaffer
- 7/9/64 - Application of Isotope Methods of Analysis. Gordon Research Conference, Tilton, N. H. - R. Schaffer
- 7/13/64 - Interpretation of Reactions in the Carbohydrate Field in Terms of Consecutive Electron Displacements. Internationale Symposium über die Chemie der Kohlenhydrate, Münster, Germany - H. S. Isbell
- 9/1/64 - Isomerization of Reducing Sugars in Alkaline Solution. ACS - Division of Carbohydrate Chemistry, Chicago, Ill. - H. S. Isbell, R. A. Peterson, H. L. Frush, and N. B. Holt
- 9/1/64 - Oxidation of Polycyclic, Aromatic Hydrocarbons Pertinent to Air Pollution. ACS - Division of Water, Air, and Waste Chemistry - Symposium on Air Pollution, Chicago, Ill. - H. S. Isbell, R. S. Tipson, and A. J. Fatiadi
- 9/1/64 - Oxidation of Polycyclic, Aromatic Hydrocarbons on Particulate Matter. ACS - Division of Water, Air, and Waste Chemistry - Symposium on Air Pollution, Chicago, Ill. - M. N. Inscoe
- 3/12/65 - Program of Organic Chemistry Section. Analytical Chemistry Division Staff Meeting, Chem. Bldg. Lecture Room. - H. S. Isbell

- 5/7/65 - Phenylhydrazono-Phenylazo Tautomerism. Bi-sectional ACS regional meeting, University of Maryland -
A. J. Fatiadi and H. S. Isbell
- 5/7/65 - Use of Solvent Isotope Effects in the Study of Pyranose—Furanose Interconversions. Bi-sectional regional ACS meeting, University of Maryland -
C. W. R. Wade

9. PERSONNEL

Organic Chemistry, H. S. Isbell, Section Chief

R. S. Tipson
H. L. Frush
R. Schaffer
C. W. R. Wade
N. B. Holt
A. J. Fatiadi
A. Cohen

E. J. McDonald, retired
L. T. Sniegowski, resigned
R. A. Peterson, transferred
J. H. Gould "
M. N. Inscoe "
E. Barron, Summer Student, terminated
T. Andrews " " LWOP

10. ACKNOWLEDGEMENTS

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